PRECLINCAL ASSESSMENT OF THE IMMUNOSUPPRESSIVE PROPERTIES OF AN ANTI-CD4 MONOCLONAL ANTIBODY (MAB) IN AN ALLOGENEIC FOETAL RAT PANCREATIC TRANSPLANTATION MODEL.

by

Christo John Frederick Muller

Dissertation approved for the degree of Doctor in Philosophy at the University of Stellenbosch

Promotor: Professor D.F. du Toit

Co-Promotor: Professor P.J. Bouic

December 2004

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work, and that I have not previously in its entirety or in part, been submitted it at any university for a degree.

Christo J.F. Muller

SIGNATURE:

DATE:

SUMMARY

Introduction

Despite advances in insulin therapy, the side effects associated with diabetes mellitus still remain. Pancreas transplantation has benefited diabetics with end-stage renal failure by reversing the diabetic state and preventing or reversing the progression of diabetes associated diseases. Currently the side effects associated with lifelong immunosuppression preclude pancreas transplantation as a viable treatment option for both type I and II diabetics.

In the laboratory, transplanted rat foetal pancreata have been shown to be able to reverse the clinical signs of streptozotocin-induced diabetes in an isogeneic model. Reversal of diabetes by allogeneic foetal rat pancreas transplantation, although possible has proved to be more difficult due to fierce rejection of the grafts and the diabetogenic effects of conventional immunosuppressants.

Aims

One of the goals, focus and intentions of this laboratory study in rodents, is to contribute new information to the scientific literature. The potential to "reverse" the diabetic state by allogeneic foetal pancreatic transplantation, was the main stimulus for this study.

Methods

Foetal pancreata of 16-18 days gestation were transplanted into a surgically prepared renal subcapsular space. Immunosuppressive protocols used to prevent rejection of the allogeneic foetal rat pancreata included donor specific transfusion (DST), cyclosporine [a calcineurin inhibitor (CsA)], mycophenolate mofetil [a purine syntase inhibitor (MMF)], and a mouse anti-rat CD4 monoclonal antibody (W3/25). Immunosuppressants were used as monotherapies and in combination.

Results

Isogeneic foetal rat pancreas transplantation resulted in the growth and development of mature insulin producing islets of Langerhans at the site of engraftment. Allogeneic foetal pancreatic transplantation without immunosuppression resulted in complete rejection of the grafts at 14 days post-transplantation.

Histological assessment of allografts at 14 and 30 days post-transplantation showed that CsA was able to prevent acute rejection in our rat models although graft scores and survival were improved if CsA was combined with MMF. Intraperitoneal anti-CD4 monoclonal injections were well tolerated, and if given daily effectively prolonged graft survival up to 30 days. Combining DST with anti-CD4 and CsA induction therapy provided long-term graft survival without daily immunosuppression. This combination, together with allogeneic foetal rat pancreas transplantation, was effective in reversing the clinical signs of experimentally induced diabetes. To my knowledge these are the first published results in which reversal of streptozotocin induced diabetes was achieved by fully MHC mismatched foetal rat pancreatic transplantation.

Conclusion

Foetal rat pancreatic transplantation is a potential source of endocrine replacement, which, with effective immunosuppression allows for the development of functional islets able to reverse the clinical signs of experimentally induced diabetes in an allogeneic rat model. An unique immunosuppressive protocol, with potential clinical relevance in the human, combines anti-CD4 mAb, CsA and DST induction therapy, which alleviates the burden of daily immunosuppression and associated side effects.

OPSOMMING

Inleiding

Ten spyte van die vordering met moderne insulienterapie bly die newe-effekte, waarmee diabetes mellitus geassosieër is, steeds 'n probleem vir diabete. Diabetiese pasiënte met eindstadium nierversaking trek geweldig voordeel uit nier-pankreasoorplantings wat die diabetes omkeer en die progressie van diabetesverwantesiektes voorkom of selfs omkeer. Die newe-effekte van lewenslange immuunonderdrukking skakel pankreasoorplanting uit as 'n lewensvatbare behandelingsopsie vir tipe I of II diabete.

In 'n streptozotosien-geïnduseerde diabetiese rotmodel kan isogeneïese fetale pankreasoorplanting die kliniese tekens van diabetes omkeer. Die omkering van streptozotosien-geïnduseerde diabetes deur allogeneïese fetale pankreasoorplanting behoort moontlik te wees indien verwerping en die diabetogeniese newe-effekte van konvensionele immuunonderdrukkers oorkom word.

Doelstellings

Een van die mikpunte, fokusse en oogmerke van hierdie laboratorium studie in knaagdiere, is om 'n betekenisvolle bydrae tot nuwe kennis in die wetenskaplike literatuur, te maak. Die potensiaal om die diabetiese toestand deur allogeneïese fetale pankeasoorplanting om te keer, was die hoof stimulus vir die studie.

Metodes

Fetale rotpankreata van 16-18 dae gestasie was in 'n chirurgies voorbereide spasie onder die nierkapsel oorgeplant. Immuunonderdrukkende protokolle, vir die voorkomming van verwerping van die allogeneïese fetale pankreasoorplantings, het donorspesifiekeoortappings (DST), siklosporien ['n kalsineurien inhibitor (CsA)], mikofenolaat mofetiel ['n purien

sintase inhibitor (MMF)] en 'n anti-rot CD4 monoklonale antiliggaam (W3/25) ingesluit. Die immuunonderdrukkers is as mono- of as kombinasieterapie gebruik.

Resultate

Isogeneïese fetale rotpankreasoorplanting het tot die ontwikkeling van volwasse insulienproduseerende eilande van Langerhans gelei, wat die kliniese tekens van streptozotosien-geïnduseerde diabetes kon omkeer.

Allogeneïese fetale rotpankreasoorplanting sonder immuunonderdrukking het tot algehele verwerping van die oorplanting binne14 dae na oorplanting gelei.

Histologiese beoordeling van die oorplantings 14 en 30 dae na oorplanting het getoon dat CsA akute verwerping van fetale pankreasoorplantings in die rotmodelle voorkom. Indien CsA met MMF gekombineer word, word die oorplantings-telling en oorlewing verbeter. Intraperitoneale anti-CD4 monoklonale inspuitings was goed verdra, en indien daagliks toegedien, het dit die oorlewing van die pankreasoorplantings effektief tot 30 dae verleng. Die kombinasie van DST, anti-CD4 en CsA induksieterapie het tot langtermyn oorlewing van die pankreasoorplantings gelei sonder verdere daaglikse immuunonderdrukking. Die induksieterapie in kombinasie met allogeneïese fetale pankreasoorplanting was effektief in die omkering van die kliniese tekens van streptozotosien-geïnduseerde diabetes in die rot. Hierdie is, sovêr ek weet, die eerste keer dat omkering van streptozotosien-geïnduseerde diabetes suksesvol met 'n volledige MHC onverenigbare allogeneïese fetale pankreasoorplanting behaal is.

Gevolgtrekkings

Fetale rotpankreasoorplanting is 'n potensiële bron vir endokrien vervangingsterapie, wat met effektiewe immuunonderdrukking tot die ontwikkeling van funksionele eilande van Langerhans lei, wat die vermoë het om die kliniese tekens van experimenteel-geïnduseerde

diabetes in 'n allogeneïese rotmodel om te keer. 'n Unieke immuunonderdrukkingsprotokol, met kliniese relevansie, kombineer DST met anti-CD4 mAb en CsA induksieterapie wat die las van daaglikse immuunonderdrukking en die geassosieërde newe-effekte van konvensionele immuunonderdrukking verlig.

ACKNOWLEDGEMENTS

I would like to thank the staff of the Departments of Anatomy and Histology, Anatomical Pathology, Medical Biochemistry and the Central Research Facility, Faculty of Health Sciences, University of Stellenbosch, and the Experimental Biology Programme at the Medical Research Council, for their expert assistance and contributions to this project.

The Harry/Doris Crossley Foundation and the Department of Anatomy and Histology for their financial support of the project.

For their contribution to the completion of this study my special thanks to the following:

My wife Nolan, children Louise and Christo, and family for their love and support.

The University of Stellenbosch, Faculty of Health Sciences, for affording me the opportunity to study at its centre of academic excellence.

Prof. Don F du Toit (M.B.,Ch.B., F.C.S.(SA), F.R.C.S.(Edin), Ph.D.(Med), D.Phil(Oxon), Head of the Department of Anatomy and Histology, for his academic wisdom, research leadership and capacity without which this project would not be possible.

Prof. P.A.B. Wranz (M.B.,Ch.B., M.Med.), Head of the Department of Anatomical Pathology (retired). For his many hours of critical reading, support, encouragement and positive outlook for which I am truly grateful.

Prof. P.J. Bouic (Ph.D.), for his scientific input and support during this study.

Prof. A.D. Beyers (deceased), AD's expert grasp of immunology laid the cornerstone of this project and he is sorely missed both as a co-researcher and a friend.

Dr. Ben J. Page (Ph.D.), for his encouragement, scientific support and input towards the completion of this dissertation.

Mr. Johannes J. Mattysen, Mr. Romeo F.C. Lyners and Ms. Elize Arendse, for their contributions and technical support without which this project could not be done.

GLOSSARY

ALG	anti-leucocyte globulin
ALS	anti-leucocyte serum
ATG	anti-thymocyte globulin
AO	Albino inbred rat strain subline of WAG rat
APC	antigen presenting cells
ATG	Rabbit-anti-human T-Lymphocyte immune serum
$CD2^+$	Pan T-lymphocyte surface molecule
$CD25^+$	CD25 specific surface molecule
$CD4^+$	CD4 specific surface molecule
$CD8^+$	CD8 specific surface molecule
CsA	cyclosporine
DA	Dark Agouti inbred rat strain
DC	dendritic cells
DEAE	diethylaminoethyl-dextran
DPX	histological mounting medium containing distrene, dibutyl
DST	donor specific transfusion
FACS	flow Cytometry
Fc	crystallizable fragment

FK506	tacrolimus
FRP	foetal rat pancreas
FRPT	foetal rat pancreas transplantation
FSC	forward scatter
GM-CSF	granulocyte monocyte colony stimulating factor
H&E	haematoxylin and eosin stain
i.m.i	intramuscular injection
i.p.i.	intraperitoneal injection
i.v.i.	intravascular injection
ICAM	intercellular adhesion molecules
ICC	immunocytochemistry
IDDM	insulin dependent diabetes mellitus
IFNγ	interferon gamma
Ig	immunoglobulin
IGF-1	insulin growth factor 1
IL	interleukine
IL-2	interleukin 2
IMPDH	inosine monophosphate dehydrogenase
IVGTT	intravenous glucose tolerance test
kV	kilovolt
L3T4	anti-mouse CD4 monoclonal antibody

LAT	linker for activation of T-cells
lck	a SRC-family tyrosine kinase
LFA	lymphocyte function associated molecule
mAb	monoclonal antibody
MHC	major histocompatibility complexes
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
MNC	mononuclear cells
NIDDM	non-insulin dependent diabetes mellitus
OX34	anti-rat CD2 monoclonal antibody
OX35	anti-rat CD4 monoclonal antibody
OX38	anti-rat CD4 monoclonal antibody
OX39	anti-rat CD25 (IL-2R) monoclonal antibody
OX8	anti-rat CD8 monoclonal antibody
PBL	Peripheral blood lymphocytes
PBS	phosphate buffered saline
PP	Pancreatic polypeptide
PVG	Black hooded (piebald) inbred rat strain
RIB5/2	anti-rat CD4 monoclonal antibody
RPMI	synthetic tissue culture medium
RPT	rat pancreatic transplantation

RT1	antigen encoded by the MHC of the rat
SD	Sprague-Dawley rat strain
SSC	Side scatter
STZ	Streptozotocin
TBS	tris buffered saline
TGF-β	transforming growth factor β
Th1	T-helper 1 CD4 T-cells
Th2	T-helper 2 CD4 T-cells
TNF-α	Tumor necrosis factor alpha
T-reg cells	T- regulatory cells
W/F	Wistar-Furth inbred rat strain
w/v	weight per volume
W3/25	mouse anti-rat CD4 monoclonal antibody
WAT	white adipose tissue
YTS 191.1	a depleting anti-mouse CD4 monoclonal antibody
ZAP-70	tyrosine kinase zeta-associated protein of 70 KDa

RESEACH OUTPUTS RELATED TO THIS PROJECT

PUBLICATIONS.

1. DU TOIT D., MULLER C., PAGE B. AND LOUW J. Foetal Rat Pancreatic Transplantation: Posttransplantation Development of Foetal Pancreatic Iso- and Allografts and Suppression of Rejection with Mycophenolate Mofetil (MMF) and Cyclosporine Based immunesuppression. Microscopy Research and Technique 1998; 43 (8): 347 –355.
2. DU TOIT D.F., MULLER C., MOUTON Y., PAGE B., MATTYSEN J., LYNERS R. AND WOODROOF C. Tacrolimus (FK506) Monotherapy Provides Potent and Significant Suppression of Allogeneic Foetal Rat pancreatic Allograft Rejection. Transplant Proc 1998; 30 (8): 4073 – 4074.
3. DU TOIT D.F., MULLER C., PAGE B., MATTYSEN J., LYNERS R. Immunosuppression With Cyclosporin A in Combination With Mycophenolate Mofetil Suppresses Rejection of Allogeneic Fetal Rat Pancreatic Allografts. Transplant Proc 1998; 30 (8): 4092 – 4093.
4. MULLER C.J.F., DU TOIT D.F., BEYERS A.D., PAGE B.J. AND MULLER N. Prolongation of Rat Fetal Pancreas Allograft Survival Using a Nondepleting Anti-CD4 Monoclonal Antibody W3/25. Transplant Proc 1998; 30 (8): 4180 – 4183.
5. MULLER C.J.F., DU TOIT D.F., PAGE B.J., LOUW J., MULLER B.J.F., WILLIAMS K., MATTYSEN J.J., AND LYNERS R.F.C. Islet Morphology and Function Following Foetal Rat Pancreatic Transplantation. Eur J Anat 2000; 4(3): 149 - 160
6. MULLER C.J.F., DU TOIT D.F., PAGE B.J., MULLER N., MATTYSEN J. AND LYNERS R. The Efficacy of Malononitrilamide 715 (MNA) as Immunosuppressant, Alone or in Combination with Cyclosporin (CSA), in Allogeneic Foetal Rat Pancreatic Transplantion. Transplant Proc 2001; 33: 2229 – 2231.

7. MULLER C.J.F., DU TOIT D.F., PAGE B.J., LOUW J., MULLER B.J.F., MATTYSEN J.J., AND LYNERS R.F.C. A Morphometric and Functional Assessment of Foetal Pancreatic Transplantation in a Syngeneic Laboratory Diabetic Rat Model. Acta Chir. Austriaca 2001; 33(174): 42.
8. MULLER C.J.F., DU TOIT D.F., PAGE B.J., MULLER N., MATTYSEN J. AND LYNERS R. Peri-Transplant Donor Specific Transfusion Combined With Anti-CD4 And Cyclosporine Induction Therapy Prolongs Foetal Rat Pancreas Allograft Survival. Transplant Proc 2002; 37(7): 2889 –2890.

PAPERS PRESENTED AT NATIONAL AND INTERNATIONAL CONGRESSES.

1. MULLER C.J.F., DU TOIT D.F., PAGE B.J., BEYERS A.D., MULLER N., MATTYSEN J. AND LYNERS R.F.C. Prolongation of Foetal Rat Pancreas Allograft Survival using a Novel non-depleting Rat Anti-CD4 Monoclonal Antibody Clone W3/25. 3rd International Conference on New Trends in Clinical and Experimental Immunosuppression, Geneva, Switzerland, February 1998.
2. MULLER C.J.F., DU TOIT D.F., PAGE B.J., MATTYSEN J., ARENDSE C. AND LYNERS R. The Efficacy of Mycophenolate Mofetil (MMF) and Malononitrilamide (MNA) As Immunosuppressants, Alone or In Combination With Cyclosporin (Csa), In An Allogeneic Foetal Rat Pancreatic Transplantion. XVIII Congress of the Southern African Transplantation Society. Mount Amanzi Lodge, Hartebeesport Dam, April 1999.
3. MULLER C.J.F., DU TOIT D.F., BEYERS A.D., PAGE B.J., MULLER N., MATTYSEN J., AND LYNERS R. Prolongation of Foetal Rat Pancreas Allograft Survival Using A Nondepleting Anti CD4 Monoclonal Antibody W3/25. XVIII Congress Of The Southern African Transplantation Society. Mount Amanzi Lodge, Hartebeesport Dam, April 1999.
4. MULLER C.J.F., DU TOIT D.F., PAGE B.J., MATTYSEN J., AND LYNERS R. The Efficacy of Malononitrilamide 715 (MNA) as Immunosuppressant, Alone or in Combination with Cyclosporin (CsA), In Allogeneic Foetal Rat Pancreatic

Transplantion. 4th International Conference on New trends In Clinical and Experimental Immunosuppression. Geneva, Switserland, February 2000.

5. MULLER C.J.F., DU TOIT D.F., PAGE B.J., LOUW J., MULLER N., MATTYSEN J. AND LYNERS R. Is Foetal Pancreas Transplantation Able To Reverse Chemically Induced Diabetes in an Isogeneic Rat Model? – A Long-term Follow-up. 30th Annual Congress of the Anatomical Society of Southern Africa. Tygerberg, April 2000.

6. MULLER C.J.F., DU TOIT D.F., PAGE B.J., MULLER N., MATTHYSEN J., LYNERS R.F.C.. Peri-Transplant Donor Specific Transfusion Combined With Anti-CD4 And Cyclosporine Induction Therapy Prolongs Foetal Rat Pancreas Allograft Survival. 5th International Conference on New Trends In Clinical and Experimental Immunosuppression. Geneva, Switzerland February 2002.

7. MULLER C.J.F., DU TOIT D.F., PAGE B.J., LOUW J., MULLER B.J.F., MATTYSEN J.J., AND LYNERS. R.F.C. A Morphometric and Functional Assessment of Foetal Pancreatic Transplantation in a Syngeneic Laboratory Diabetic Rat Model. 8th Congress IPITA, 12 –15 June 2001, Innsbruck.

8. MULLER C.J.F., RUTTEN C., DU TOIT D.F., PAGE B.J. AND LYNERS R.F.C. Allogeneic Foetal Rat Pancreatic Transplantation in a Tolerant Laboratory Diabetic Rat Model. 31st Surgical Research Society Symposium, Pretoria, July 2003.

9. MULLER C.J.F., RUTTEN C., DU TOIT D.F., PAGE B.J. AND LYNERS. R.F.C. Donor Specific Transfusion In Combination With Cyclosporine And Anti-CD4 mAb Induction Therapy Results In Foetal Rat Pancreatic Allograft Acceptance - Are $CD2^+CD4^+$ Regulatory Cells Involved? XX Southern African Transplantation Society Congress, Magaliesburg, September 2003.

10. MULLER C.J.F., RUTTEN C., DU TOIT D.F., PAGE B.J., LYNERS R.F.C. Donor specific transfusion in combination with cyclosporine and anti-CD4 MAB induction therapy results in foetal rat pancreatic allograft acceptance - are $CD2^+CD4^+$ regulatory cells involved? 47th Academic Yearday, August 2003, University of Stellenbosch, Faculty of Health Sciences, Tygerberg.

11. MULLER C.J.F., RUTTEN C., DU TOIT D.F., PAGE B.J. AND LYNERS R.F.C. Assessment of allogeneic foetal rat pancreatic transplantation in a tolerant laboratory

diabetic rat model. 34th Annual Congress of the Anatomical Society of Southern Africa. Durban, April 2004.

POSTER PRESENTATIONS.

1. MULLER C.J.F., DU TOIT D.F., BEYERS A.D., MOUTON Y.M., MULLER N., LYNERS R.F.C. AND MATTYSEN J. Prolongation of Rat Foetal Allograft Survival using a Novel non-depleting Rat Anti-CD4 Monoclonal antibody W3/25. 41st Academic Yearday, Faculty of Medicine and Dentistry, University of Stellenbosch, August 1997.
2. MULLER C.J.F., DU TOIT D.F., MOUTON Y.M., MULLER N. BEYERS A.D., LYNERS R.F.C. AND MATTYSEN J. Characterization Of The Intragraft Mononuclear Cell Infiltrate After Sub-Capsular Allogenic Foetal Pancreas Transplants In Rats. 41st Academic Yearday, Faculty of Medicine and Dentistry, University of Stellenbosch, August 1997.
3. MULLER C.J.F., DU TOIT D.F., MOUTON Y.M. AND BEYERS A.D. A Comparison of Foetal Pancreas Allograft Immunemodulation in Rats, using a Non-depleting Anti-CD4 Monoclonal Antibody (W3/25) to Cyclosporin. 37th Annual Congress of the Federation of South African Societies of Patology, Cape Town, June/July 1997.
4. DU TOIT D.F., MULLER C.J.F., MOUTON Y.M., MATTYSEN J. AND LYNERS R.F.C.. Polongation of Foetal rat pancreatic allograft Survival with Tacrolimus (FK506) and Cyclosporin (CsA). Surgical Research Society of Southern Africa Congress, Stellenbosch, July 1997.
5. DU TOIT D.F., MULLER C.J.F., PAGE B.J., BEYERS A.D., MULLER N., MATTYSEN J. AND LYNERS R.F.C. Immunosuppression With Cyclosporin A In Combination With MycophenolateMofetil Suppresses Rejection Of Allogeneic Foetal Pancreatic Allografts. 3rd International Conference on New Trends in Clinical and Experimental Immunosuppression, Geneva, Switzerland, February 1998.

6. D.F. DU TOIT, C.J.F. MULLER, B.J. PAGE, A.D. BEYERS, N. MULLER, J. MATTYSEN AND R.F.C. LYNERS. Tacrolimus (FK506) Monotherapy Provides Potent And Significant Suppression Of Allogeneic Foetal Rat Pancreatic Allograft Rejection. 3rd International Conference on New Trends in Clinical and Experimental Immunosuppression, Geneva, Switzerland, February 1998.

7. MULLER C.J.F., PAGE B.J., DU TOIT D.F., MULLER N., LYNERS R.F.C. AND MATTYSEN J. Die Vestiging Van 'N Immuunbevoorregte Area Onder Die Nierkapsel In 'N Rotmodel Vir Fetale Pankreas Oorplanting. 42nd Academic Yearday, Faculty of Medicine and Dentistry, University of Stellenbosch, August 1998.

8. C.J.F. MULLER, D.F. DU TOIT, B.J. PAGE, C. WOODROOF, N. MULLER, J. MATTYSEN, C. ARENDSE EN R. LYNERS. Kan Fetale Pankreasoorplanting Chemies Ge-Ïnduseerde Diabetes In 'N Isogeneïese Rotmodel Omkeer? – 'N Langtermyn Opvolg. 43rd Academic Yearday, Faculties of Medicine and Dentistry, University of Stellenbosch, August 1999.

9. MULLER C.J.F., DUTOIT D.F., PAGE B.J., MATTYSEN J., ARENDSE C. AND LYNERS R.. The Efficacy of Mycophenolate Mofetil (MMF) and Malononitrilamide (MNA) As Immunosuppressants, Alone or in Combination with Cyclosporin (CsA), In Allogeneic Foetal Rat Pancreatic Transplantion. 43rd Academic Yearday, Faculties of Medicine and Dentistry, University of Stellenbosch, 18-19 August 1999.

10. MULLER C.J.F., DU TOIT D.F., PAGE BJ., MATTHYSEN J.J. AND LYNERS R.FC. The efficacy of mycophenolate mofetil (MMF) in preventing acute rejection following withdrawal of cyclosporine (CsA) from cyclosporine and mycophenolate mofetil combination immunosuppression in a rat foetal pancreas allograft transplant model. 44th Academic Yearday, Faculties of Medicine and Dentistry, University of Stellenbosch, August 2000.

11. MULLER C.J.F., DU TOIT D.F., PAGE B.J., MATTYSEN J. AND LYNERS R. The efficacy of malononitrilamide 715 (MNA) as immunosuppressant, alone or in combination with cyclosporin (CsA), in allogeneic foetal rat pancreatic transplantion. 44th Academic Yearday, Faculties of Medicine and Dentistry, University of Stellenbosch, August 2000.

12. MULLER C.J.F., DU TOIT D.F., PAGE B.J., LOUW J., MULLER N., MATTYSEN J. AND LYNERS R. A Morphometric and Functional Assessment of Foetal Rat Pancreatic Transplantion in a Syngeneic Laboratory Diabetic Rat Model. 45th Academic Yearday, Faculty of Health Sciences, University of Stellenbosch, 22-23 August 2001.

13. C.J.F. MULLER, DU TOIT D.F., PAGE B.J., MATTYSEN J. AND R LYNERS. Characterization of Foetal Pancreas Allograft Lymphocytic Infiltrates Following Immunosuppression with Anti-CD4 Antibody (W3/25) and Corticosteriods (Prednisoslone). 45th Academic Yearday, Faculty of Health Sciences, University of Stellenbosch, 22-23 August 2001.

14. MULLER C.J.F., DU TOIT D.F., PAGE B.J., LABUSCHAGNE B.C.J., MULLER N., MATTYSEN J.J. AND LYNERS R.F.C. A comparison of endocrine development following syngeneic and allogeneic foetal rat pancreas transplantation. 32nd Annual Congress of the Anatomical Society of Southern Africa. Durban, April 2002.

15. MULLER C.J.F., W. VORSTER, B.C.J. LABUSCHAGNE, D.F. DU TOIT, B.J. PAGE, N. MULLER, J. MATTYSEN AND R. LYNERS. A histological and morphometric assessment of islet development following isogeneic and allogeneic foetal rat pancreas transplantation. Proceedings XVII International Symposium on Morphological Sciences 2002; Sept: 336.

16. MULLER C.J.F., GELDENHUYS K.M., DU TOIT D.F., PAGE B.J., MATTYSEN J.J., AND RFC. LYNERS. Early endocrine development of transplanted foetal rat pancreata. 33rd Annual Conference of the Anatomical Society of Southern Africa. Golden Gate, Bloemfontein 7 - 10 April 2003.

17. MULLER C.J.F., RUTTEN C., DU TOIT D.F., PAGE B.J., LYNERS R.F.C. Allogeneic foetal rat pancreatic transplantation in a tolerant laboratory diabetic rat model. 47th Academic Yearday, Faculty of Health Sciences, University of Stellenbosch, August 2003.

18. MULLER C.J.F., DU TOIT D.F., PAGE B.J., GELDENHUYS K.M., MATTYSEN J., LYNERS R.F.C. Early endocrine development of transplanted foetal rat pancreata. 47th Academic Yearday, Faculty of Health Sciences, University of Stellenbosch, August 2003.

Table of Contents

CHAPTER 4

CHAPTER 5

CHAPTER 6

List of Tables

List of Figures

Chapter 1

DIABETES MELLITUS - THE PROBLEM

Introduction and History

Diabetes, a complex group of diseases with hyperglycaemia a characteristic feature, has been known and researched since antiquity, but still remains a worldwide health problem. Originally it was thought that diabetes was a disease of the kidneys, a concept which was further entrenched with the discovery of the sugar in, or sweetness of the urine by Thomas Willis 1621 – 79 [1]. The discovery that a pancreatectomy causes diabetes by Von Mering and Minkowsky in Strasbourg in 1889, lead to our present understanding of the cause of diabetes[1].

The discovery of insulin by Frederick Banting, Charles Best, Macleod and James Collip in 1921, has not only saved the lives of millions of diabetics but has also greatly improved the prognosis and quality of life of diabetics worldwide[1]. However, despite this lifesaving contribution of insulin in preventing acute metabolic death by ketoacidosis, it soon become clear that diabetes is not simply a "sugar problem" but that the chronic complications of the disease persist even with good insulin and diet control[2]. Subcutaneous injection of insulin presents its own problems with hyperinsulinaemia a further complication linked to the development of macroangiopathies[2].

Diabetes is not a single disease but includes a group of diseases caused by a wide range of known and unknown factors including various virusses, chemical toxicity and pollutants, genetic predisposition, geographic locality and diet. Clinically there are two types of "Diabetes" i.e. Type I insulin dependent diabetes mellitus (IDDM) and Type II non-insulin dependent diabetes mellitus (NIDDM), being characterized by the absence or presence of plasma insulin respectively.

Insulin and insulin receptors

The availability of insulin and target organ/cell insulin receptors are the major role players in the maintenance of euglycaemia. Insulin, a hormone coded by a gene on chromosome 11 is produced as preproinsulin, converted to insulin by removal of the connecting peptide (C-peptide) in the Golgi apparatus by enzymes with trypsin and carboxypeptidase activity. Insulin then complexes with Zn to form micro-crystals in the typical secretion granules seen in active β cells. Insulin release from the β-cells is stimulated by glucose, requiring glucose metabolism and ATP resulting in the closure of the K^+ channels and increased inflow of Ca^{++} into the cell[3]. Following release, insulin binds to the insulin receptor of the target cell. The insulin receptor is coded by genes on chromosome 19 and consists of two α subunits covalently bound to two β subunits situated on the surface of the target cell. Binding of insulin to its receptor on the target cell results in phosphorylation and autophosphorylation of tyrosine residues inhibiting glycogenolysis. The insulin-insulin receptor complex is then internalized, the insulin degraded and the insulin receptor is then recycled to the cell surface[3]. Thus any defect in insulin, insulin secretion or the availability of insulin receptors cause an increase in blood glucose levels and diabetes.

Diabetes mellitus onset and progression

IDDM

IDDM is a multifactorial disease leading to selective destruction of the insulin secreting β-cells by an autoimmune process induced by environmental factors in early life, even as early as intra-

uterine life[4]. In Western industrialized countries IDDM is the second most common chronic childhood disease after asthma[3].

IDDM has a sudden onset, normally seen in younger patients, peaking in the mid-teens, caused by the destruction of the insulin producing endocrine β-cells either by direct cytotoxicity or by autoimmune destruction of these β-cells. The disease manifests itself at the stage when approximately 80% of the β-cells are destroyed which could take as long as 5 –15 years[4]. Typically symptoms at this late stage of the disease include hyperglycaemia and/or ketoacidosis. The progression of the disease is rapid requiring medical intervention. Causes of IDDM are more obscure with a genetic predisposition along with environmental factors, being the most obvious. Genetically, individuals with HLA class II DR3,4; DQ2,8 and class I A24 alleles are at increased risk[5,6,7,8]. Of interest is the geographic distribution of childhood IDDM, showing a general increase in incidence from the equator towards the two poles. Northern Europe, Finland, Sweden and Scotland in particular, show a rapid increase in the incidence of IDDM as compared to Southern European countries. Finland has the highest incidence of childhood diabetes at 30/100 000 per year compared to the 1/100 000 per year seen in Japan. Genetic factors are certainly thought to be a major contributing factor, with various environmental factors contributing to the rapid increase of incidence of childhood IDDM in Northern Europe. A study comparing identical twins of diabetic parents to non-identical twins of diabetic parents found that 58% of identical twins, compared to 17%, were concordant for diabetes. Insulin dependent diabetes mellitus is often found in family clusters but this is not conclusive proof of genetic predisposition as families share a similar environment and only approximately 5% of diabetics have a familial history of IDDM[4].

Epidemiologists have been able to show a seasonal variation in the onset of acute IDDM which was confirmed in both the Northern and Southern hemispheres with a higher onset in the autumn and winter months [9]. Viral infection, alone or in combination with other environmental factors, is certainly one of the most likely candidates responsible for these seasonal variations[1]. Viral infections have however rarely been shown to be solely pathogenic for IDDM although congenital rubella is associated with an increased risk of IDDM with a similar onset age and immunogenic predisposition to idiopathic IDDM[4].

NIDDM

NIDDM normally has a more chronic onset manifesting itself in later life and it is estimated that 10% of Europeans above the age of 75 years may be diabetic with 45% of men in the age group 75 –79 years in east Finland suffering from NIDDM[3]. Except for a genetic predisposition for developing NIDDM such as the Pima Indians of Arizona with an incidence of 30 –35%, evidence clearly shows that urbanization and industrialization along with a Westernized diet and lifestyle are the main factors contributing to NIDDM reaching endemic proportions.

NIDDM is caused by either an insufficient β-cell mass, especially prevalent in obese patients or insensitivity to insulin[3]. Strong evidence exists that NIDDM in later life could be due to the loss of β-cells by an aborted IDDM autoimmune process[1]. Malnutrition during foetal development and early life has been show to increase the risk of developing NIDDM in later life especially when associated with obesity in later life. This phenomenon has been termed the "thrifty phenotype" and is probably associated with a lower β-cell mass caused by the early malnutrition[9]. A change of diet especially from a rural to a more Westernized diet containing

highly refined carbohydrates, fat and alcohol, is associated with a higher incidence of diabetes, as seen in the Nauruans, Pimas and Aboriginal populations in New South Wales[9]. The estimated heritability of NIDDM is around 82% in a particular environment and changing the environment dramatically influences the genetic index even between twins[3].

Complications associated with diabetes.

Diabetes is a major and increasing world health problem affecting approximately 130 million people[10]. Industrialization has seen non-communicable diseases increase dramatically with cancer, arterial disease and diabetes the leading causes of early death. Diabetes is the leading cause of blindness during working life and the second most common cause of kidney failure. Macro- and microvascular disease are common complications, often resulting in amputations, stroke and heart attacks in diabetics. The life expectancy of diabetics, on average, is reduced by a decade[3]. The true cost of diabetes to the patient both in financial terms and in life expectancy is astronomic with costs rising as medical and life insurance premiums are loaded to counter the long-term complications. People suffering from IDDM are excluded from many employment opportunities e.g. becoming airline pilots[1]. Although insulin treatment has been refined and tight control of blood glucose levels is possible though a controlled carbohydrate diet combined with a balanced insulin treatment regimen, complications do occur and are varied resulting in the high treatment costs of arterial sclerosis, retinopathy leading to blindness, renal failure and dialysis and debilitating amputations. These secondary complications still are particularly common in underprivileged third-world nations and races. Diabetics remain uninsurable by insurance companies or pay enormous premiums. The cost of insulin is staggering and most medical-aids refuse cover for diabetics, even when excellently controlled. Although there is a good correlation

between tight diabetic treatment control as measured by the glycaemic index, complications such as retinopathy, nephropathy and neuropathy still progress albeit at a slower rate. Sudden changes in diabetic control even for the better may accelerate the progression of the disease.

BIBLIOGRAPHY

[1] Watkins PJ. Diabetes and its management. Oxford: Blackwell Scientific Publications; 1993.

[2] Mandel TE. Fetal islet transplantation. Transplant Proc 1992; 24: 1996-1997.

[3] Leslie RDG. Metabolic Changes in Diabetes. Eye 1993; 7: 205-208.

[4] Hitman GA. The immunogenetics of insulin-dependent diabetes. Eye 1993; 7: 209–213.

[5] Caillat-Zucman S, Garchon HJ, Timsit J, Assan R, Boitard C, Djilali-Saiah I, Bougneres P, Bach JF. Age-dependent HLA genetic heterogeneity of type 1 insulin-dependent diabetes mellitus. J Clin Invest 1992; 90: 2242-50.

[6] Tait BD, Harrison LC, Drummond BP, Stewart V, Varney MD, Honeyman MC. HLA antigens and age at diagnosis of insulin-dependent diabetes mellitus. Hum Immunol 1995; 42: 116-122.

[7] Honeyman MC, Harrison LC, Drummond B, Colman PG, Tait BD. Analysis of families at risk for insulin-dependent diabetes reveals that HLA antigens influence progression to preclinical disease. Mol Med 1995; 1: 576-582.

[8] Hoogwerf BJ, Rich SS, Barbosa JJ: Meal-stimulated C-peptide and insulin antibodies in type I diabetic subjects and their nondiabetic siblings characterized by HLA-DR antigens. Diabetes 1985; 34: 440-445.

[9] Williams DRR. Epidemiological and Geographic Factors in Diabetes. Eye 1993; 7: 202-204.

[10] Burridge PW, Shapiro AM, Ryan EA, Lakey JR. Future trends in clinical islet transplantation. Transplant Proc 2002; 34(8): 3347-8.

Chapter 2

PANCREAS TRANSPLANTATION

History of pancreas transplantation

Pancreas transplantation as an endocrine replacement therapy has a long history with the first record of a pancreas transplant being performed in 1893, 29 years prior to the discovery of insulin, by Drs P.W.Williams and Harsant of the Bristol Royal Infirmary. They transplanted three pieces of freshly slaughtered sheep pancreas sub-cutaneously into a 15 year old diabetic. The boy died three days after the transplantation. Histology of the grafts showed only fibrous stroma[1,2,3]. Ssobolew and Allan recommended pancreas transplantation as a treatment for diabetes in 1902. Notes published by Pybus in the Lancet in 1924 described subcutaneous transplants of slices of human pancreas into two diabetic patients[2]. Both of the patients rejected their grafts but one showed a temporary reduction of glycosuria, a remarkable result considering that no immunosuppression was available and the strong rejecting potential of the skin. Luisada (1927), as described by Downing in a historical review of pancreatic islet transplantation, transplanted duct-ligated baboon pancreas under the tunica vaginalis of two young diabetics, but without any beneficial effect[2].

These early attempts at pancreas transplantation although imaginative and ambitious were doomed to failure by rejection. Autogeneic transplantations, on various laboratory animals, were however successful and contributed to the understanding of diabetes. Minkowski, (1892 and 1893 as quoted by Downing 1984), performed auto-transplants of vascularized uncinate processes of canine pancreas under the skin. He then performed a pancreatectomy of the remaining intra-abdominal pancreas. The dogs did not develop fatal diabetes. Downing further describes that skeptics like Pfluger proposed that the transplanted segments were functional because they were in fact still connected to and innervated by the duodenum. Subsequent studies

by Hedon and Lombroso in 1910 (as quoted by Dowling 1984) showed that denervated segmental pancreas autografts were able to prevent diabetes in pancreatectomized animals thus establishing a relationship between the pancreas, diabetes and transplantation of pancreatic tissue[2].

It must be appreciated that prior to the insulin era, transplantation was regarded as the only viable treatment. Intrasplenic transplantation of pancreatic tissue gained favour due to the hypothesis of pancreatic internal secretion. Early attempts by Allesandri (1913 as quoted by Dowling 1984), Ottolenghi (1901 as quoted by Dowling 1984) and Kyrle (1908 as quoted by Dowling 1984) to transplant fragments of dog pancreata into the splenic pulp were unsuccessful. Kyrle was able to transplant vascularised pancreatic segments into the spleen of dogs and guinea pigs and could show survival of the grafts after he ligated the pedicles. Unfortunately he did not do any functional studies[2]. Pratt and Murphy were however more successful with intra-splenic autografts of the vascularized uncinate process, which they then ligated after 22 days and removed the rest of the pancreas. The graft survived for 209 days with the dog remaining insulin free for that period. The urine glucose levels were monitored and remained between 0% and 7% [2].

The search for an ideal site for pancreas transplantation was actively pursued around the turn of the century with direct intrahepatic transplants of pieces of pancreas done by Alessandri (1896 as quoted by Dowling 1984) and Ottolenghi (1901 as quoted by Dowling 1984) allotransplanting pieces of guinea-pig pancreata into the liver, spleen, peritoneal cavity and subcutaneous tissues. All these grafts showed necrosis of the grafts, within 2 days. Dowling further describes how Badile did a comparative study on various transplantation sites using both allograft and

autografts. He transplanted pieces of cat pancreas into the liver, spleen, greater omentum, retroperitoneal connective tissues and gastric mucosa. This study confirmed, what had been the pattern with pancreatic transplantation throughout, namely that allografts were rejected rapidly, but autografts were able to survive. In his study autogeneic grafts into the gastric submucosa survived for 105 days [2].

By the late 1920's it was established that autografts could survive in various sites provided the grafts were vascularised. It was also established that the exocrine portion of the grafts negatively affected graft survival. Various attempts were made to reduce exocrine secretion from the graft. Thiroloix in 1892, injected the pancreatic ducts with oil and lamp-black to suppress exocrine secretion and showed that splenic autografts although fibrosed, were able to maintain normoglycaemia. In 1929 Brancati employed duct-ligation of canine pancreas autografts into the greater omentum. These autografts survived for 28 and 34 days. Histology showed normal islets with atrophied exocrine acini [2].

Pancreas transplantation

The demand for clinical pancreatic transplantation has grown rapidly following the first reported clinical segmental duct-ligated graft that was transplanted simultaneously with a kidney by Kelly et al. of the University of Minnesota in 1966 [4 5]. Lillehei et al. followed with 14 pancreas transplantations between 1966 and 1973 and were the first to transplant whole pancreaticoduodenal grafts. Groth et al. in Stockholm did a large series of enteric-drained grafts in 1973. The concept of urinary drainage was introduced by Gliedman et al. in 1971 and modified by Sollinger *et al.* at the university of Wisconsin in 1982 by a direct anastomosis of the pancreatic duct to the bladder. Nhiegm et al. at the University of Iowa further refined the

technique by leaving a segment of duodenum attached to the pancreatic-duct to make anastomosis to the bladder easier.

The Report of the International Pancreas Transplant Registry 1991 shows that almost all the pancreas transplants performed in the USA between 1986 and 1991 were by the bladder drainage technique (94% of the total, n = 1224) while in Europe less than half the transplants were by the bladder drainage technique (42% of the total, n = 313). The bladder drainage technique has a smaller technical failure rate of 13% and 17%, in the USA and Europe respectively. The registry shows first year graft survival by this technique to be at 68% and 54% respectively. The intestinal drainage technique was the only other duct-management technique employed by American researchers (63 patients) in the period. The technical failure rates using this technique are higher with 28% and 25% in USA and Europe respectively. Duct injection was only done in Europe with a functional graft first year survival rate of 56% [6].

The aims of pancreas transplantation are firstly to establish normoglycaemia and insulin independence in the diabetic patient and secondly to prevent, halt or reverse the progression of secondary complications of diabetes. The object of pancreas transplantation is to improve the quality of life and this must be the only criterion in the selection of transplant patients. Pancreas transplantation, as with other transplants, commits the patient to life-long immunosuppression at this time. The side effects of current immunosuppressive drugs could outweigh the advantage of the pancreas transplant even if the patient is euglycemic. Diabetic patients that would most certainly benefit from pancreas transplantation are patients with end stage renal failure due to diabetic nephropathy, requiring renal transplantation[4]. These patients are obligated to take immunosuppressives to maintain the renal graft. More than 75% of pancreas transplants reported

during 1986 – 90 were simultaneous kidney/pancreas transplants with a 68% first year survival and patients were dialysis free and insulin independent[2].

There are patients whose problems with diabetes severely impacts on their quality of life. Diabetic patients with autonomic or somatic neuropathy have a high mortality. Pancreas transplantation has been shown to improve the condition of these patients as measured by their increase in nerve conduction velocities and autonomic index[7]. Reversal of severe secondary complications in diabetic patients by pancreas transplantation has been somewhat disappointing, as shown by the Minnesota series with the continued progression of retinopathy in 30% of patients with functioning grafts a figure similar to that of the patients with failed grafts. After 3 years however, no further progession occurred in the recipients with funtioning grafts. However, 70% with failed transplants advanced to a higher grade within 5 years[5].

The recurrence of diabetic nephropathy occurrs in nearly half of the kidneys transplanted without pancreas in uraemic diabetic patients[8]. A successful pancreas transplant influences the progression and even reverses the course of diabetic nephropathy. Ten year follow up biopsies showed that glomerular and tubular membrane thickness and mesangial fraction volume of the glomerulus had decreased and returned to normal following pancreas transplantation[5].

The Munich group (1992) demonstrated a beneficial effect on the microcirculation following a successful pancreas transplant and concluded that if the pancreas transplants were performed earlier in the course of the disease secondary complications might be prevented[4].

Studies have shown that the quality of life is better in uraemic recipients who received both kidney and pancreas compared to those patients who received kidney transplants alone. Patients

who received single pancreas transplants were also overwhelmingly positive regarding their improved quality of life, despite the side effects of immunosuppression. Both groups indicated that they would have a retransplant if the transplanted pancreas failed[4].

The complex surgery required to perform vascularized whole pancreas transplants, which includes unnecessary exocrine acinar tissue, has boosted research into the transplantation of islet-rich tissues.

Islet transplantation

Two approaches are adopted namely, transplantation of isolated islets and foetal or neonatal transplantation.

The development of islet isolation and transplantation has been hampered by the technical difficulties of isolating viable mammalian islets in sufficient numbers to have any effect on blood glucose levels. Younoszai et al. were the first to successfully isolate rat islets and were able to ameliorate chemically induced diabetes by intraperitoneal transplantation of isolated islets [9].

Two methods of islet separation from pancreatic tissue have been employed. Microdissection techniques, as pioneered by Norberg in 1964 (as quoted by Dowling 1984) was found to be too traumatic, yielding only small numbers of viable islets. Moskalewski (as quoted by Dowling 1984) was the first to employ collagenase to digest chopped guinea-pig pancreas and although the collagenase destroyed some islets, separation of the islets from the acinar exocrine tissue was possible. The technique was further refined by Lacy and Kostianovsky in 1967 (as quoted by Dowling 1984) by intraductal distention prior to digestion with collagenase. Islets were then

separated from the digested pancreatic tissue by sucrose gradient centifugation. This modification secured approximately 300 intact islets from a single rat pancreas. Further refinements using ficoll gradients by Sorenson in 1968 improved the islet yield and paved the way for human islet separation in 1971 [2].

In 1972, Ballinger and Lacy succeeded in the long-term amelioration of streptozotocin induced diabetes in rats by intraperitoneal and intramuscular isotransplantation of between 400 and 600 separated islets. Islet transplantation was boosted when Kemp showed that intraportal embolisation of between 400 – 600 rodent islets could completely normalize blood and urine glucose levels within two days of implantation due to the increased viability of islets with an immediate blood supply together with a normal physiological route of insulin secretion [10].

Human clinical results, using this technique have, despite early optimism, been disappointing. Three hundred and five adult islet allografts had been performed in 38 institutions by December 1995, and of these patients only 39 were functionally insulin independent at one month post-transplantation and of these 39, only 24 were still insulin independent at 12 months. Only one patient was still insulin independent at four years [11].

Analysis of c-peptide negative patients receiving islet transplants, in the period 1990 – 94, showed that of 96 patients known to be c-peptide negative prior to transplantation, 27% had basal c-peptide levels in excess of 1 ng/ml at one year and of these only seven percent were insulin independent [11]. If the only measure of success is insulin independence then these results are dismal indeed; patients with persisting c-peptide levels, although not sufficient to be insulin independent, benefitted by requiring lower doses of insulin and improved glycaemic control leading to a decrease in complications[12]. Guidelines put forward by the International Islet

Transplant registry for attaining insulin independence by islet transplantation are the following: harvesting of islets from pancreata within eight hours with adequate preservation; transplantation of > 6000 islets, with a mean diameter of at least 150 μm, per kilogram (kg) body weight; high level of immunosuppression using T-cell antibodies anti-leucocyte globulin (ALG) or anti-thymocyte globulin (ATG).

The above requirements demonstrate the problems encountered in islet transplantation especially regarding the obtaining of sufficient islet mass to ameliorate diabetes.

The introduction of the Edmonton Protocol, a steroid-free immunosuppressive regimen, together with optimal islet mass transplantation of 11000 islets/kg has dramatically altered the outcome of islet transplantation. Typically this would require a minimum of three donor pancreata. Currently the Edmonton series includes 35 patients of whom all achieved insulin independence post-transplantation, with the longest insulin-free follow-up being more than three years. Currently their one year insulin independence rate is 87% [1].

Foetal pancreas transplantation

A viable alternative to adult islet transplantation is foetal pancreas transplantation[13,14]. The possiblity of using foetal pancreatic tissue was first raised by Lazarow et al. in 1973[15]. The early development of the endocrine tissue compared to the exocrine pancreas tissue provides an opportunity for the preferential exploitation of foetal pancreas as a source of endocrine rich tissue with a high growth potential[16,17]. Although many clinical foetal pancreas transplants have been done in Russia, China, Europe and the USA[12], the subsequent ban on the clinical use of foetal tissue, which still remains in force, has prevented further studies in the USA and Europe[12,18,19].

Lafferty et al. transplanted human foetal tissue under the kidney capsule of donor kidneys that were transplanted into diabetic patients scheduled for kidney transplantation because of their uremia[20]. Although none of the patients became insulin independent all required less insulin following the transplant. Tissue taken from the grafts showed the development of islet tissue, which could be stained for the presence of insulin[12].

Preclinical experimentation using the rodent and other animal models has paved the way for rapid and effective transplantation once the practical, political and ethical concerns have been overcome. The reversal of streptozotocin-induced diabetes in rats by renal subcapsular transplantation of foetal pancreata has been demonstrated by Brown *et al.* in 1976 and 1981, Mullen 1976 and Spence et al. in 1981[17,21,22,23].

Brown and Mullen showed that one syngeneic rat foetal pancreas of between 17 and 17½ days gestation, engrafted under the kidney capsule of a Lewis rat, rendered previously diabetic by intravenous injection of 72.5 mg/kg streptozotocin, could reverse insulin dependence and ameliorate the diabetes. Brown confirmed the enormous growth potential of foetal pancreatic tissue and calculated that a single foetal pancreas engrafted under the kidney capsule could develop to 22% of the adult beta cell component. Normoglycaemic values were maintained by the single graft for about 4 months. Removal of the grafts returned the animal to the pretransplant levels of hyperglycaemia, polyuria and glycosuria. Brown has emphasised the following points: There is a 4000-fold increase in the insulin content of the grafts within the four month period from 0.2 mU to 818 mU which is 22% of the insulin content of normal rats and since the removal of approximately 90% of the pancreas is required to induce diabetes, it may be concluded that one foetal pancreas could reverse the diabetes and maintain euglycaemia.

Another interesting point regarding beta cell proliferation is the stimulating effect of superphysiological glucose levels on beta cell mass. McEvoy et al. found the beta cell mass of transplanted foetal pancreata to be the same in normal and untreated diabetic rats but found a three fold increase if transplanted diabetic rats received insulin post-transplantation[24,21]. Transplantation of foetal pancreata into normal non-diabetic rat for two weeks, followed by removal of the kidney and the undisturbed grafts and transplanting them into diabetic rats resulted in improved graft function when compared to direct engraftment into a diabetic rat. Grafts that were maintained in the normal rat for 5 weeks showed an impaired release of insulin on glucose stimulation. It would thus appear that maintenance of normoglycaemic conditions is important for growth, development and function of transplanted foetal pancreata [25].

Brown found that the basal and glucose stimulated blood insulin levels of the transplanted rats were moderately higher than normal. This, he proposes, is due to the altered venous drainage of the kidney subcapsular engraftment site. Dye injection studies have shown that the main venous drainage of foetal pancreas transplants from the kidney subcapsular site is into the renal vein and then into the inferior vena cava thus bypassing the liver [25]. The liver usually extracts approximately 50% of the insulin present in the portal vein with each pass. Madison et al. showed that if ^{131}I-labelled insulin is injected into the portal vein, 51% of the insulin is recovered in the liver while only 27% of insulin is recovered in the liver if the labeled insulin is injected into a peripheral vein[21]. The effect of the higher insulin levels, in the longer term, is not known. Rerouting the blood flow by shunting the renal vein end to side with the portal vein resulted in an immediate return of the insulin levels to normal, blood glucose clearance was improved and a normal insulin response was seen following intravenous injection [25]. Brown et al. showed that pancreata of between 17½ and 18 days were most suitable with six foetal

pancreata able to reverse diabetes in 93% of cases while four pancreata reversed diabetes in 73% of the cases[25].

Histological evaluation of the grafts at one week showed groups of cells with beta cell staining characteristics and exocrine tissue, which progressively degenerated. At one to two months endocrine tissue was well formed, the exocrine portion was limited to ducts and connective tissue and adipose tissue surrounded the structures. Electron microscopy revealled both α- and β-cells, and that the β-cells were often partly degranulated indicating active insulin secretion[25,26].

Metabolic studies done on successful transplanted diabetic rats showed, apart from a slightly elevated plasma insulin level, that other metabolic parameters (enzymes of the glycolytic pathway, glucokinase and pyruvate kinase which are severely depressed in the diabetic liver) had returned to normal values. Glucose-6-phosphate dehydrogenase and citric lyase, which are involved in lipid metabolism and fructose 1,6-diphosphatase and glucose-6-phosphatase which are involved in gluconeogenesis all returned to normal values [25,27].

Allogeneic foetal pancreas transplantation has not had the same success. Originally foetal pancreata were thought to be less immunogenic due to the immunologically immature nature of the grafts but in fact were found to be very antigenic and were fiercely rejected without effective immunosuppression [12].

BIBLIOGRAPHY

[1] Williams P.W. Notes on diabetes treated with extract and by grafts of sheep's pancreas. Br. Med. J. 2: 1303, 1894. Reference in Downing R. World J Surg 1894; 8: 137-142.

[2] Downing R. Historical review of pancreatic islet transplantation. World J Surg 1984; 8: 137-142.

[3] Burridge PW, Shapiro AM, Ryan EA, Lakey JR. Future trends in clinical islet transplantation. Transplant Proc 2002; 34(8): 3347-8

[4] Sutherland DER. Pancreatic transplantation: state of the art. Transplant Proc 1992; 24: 762-766.

[5] Sutherland DER, Gruessner RWG, Dunn DL, Matas AJ, Humar A, Kandraswamy R, Mauer S.M, Kennedy WR, Goetz FC, Robertson RP, Gruessner AC, Najarian JS. Lessons learned from more that 1000 pancreas transplants at a single institution. Annals of Surgery 2001; 233 (4): 463 - 501.

[6] Sutherland DER. Report from the international pancreas transplant registry. Diabetologia 1991; 34: 28-39.

[7] Navarro X, Kennedy WR, Loewenson RB, Sutherland DER. Influence of pancreas transplantation on cardiorespiratory reflexes, nerve conduction, and mortality in diabetes mellitus. Diabetes 1990; 39(7): 802-6.

[8] Mauer SM, Barbosa J, Vernier RL. Development of diabetic vascular lesions in normal kidney transplanted into patients with diabets mellitus. N Engl J Med 1976; 295: 916 - 920.

[9] Younoszai R, Sorenson RL, Lindall AW. Homotransplantation of isolated pancreatic islets. Diabetes 1970; 19 (1): 406.

[10] Kemp CB, Knight MJ, Scharp DW, Balinger WF, Lacy PE. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia 1973; 9: 486-491.

[11] International transplant registry. 1996; 6.

[12] Lafferty KJ, Hao L. Fetal pancreas transplantation for treatment of IDDM patients. Diabetes Care 1993; 16: 383 – 386.

[13] Sutherland DER, Matas AJ, Steffes MW, Najarian JS. Infant human pancreas. A potential source of islet tissue for transplantation. Diabetes 1976; 25(12): 1123-8.

[14] Mandel TE. Organ culture of foetal mouse and foetal human pancreatic islets for allografting. Diabetes 1982; 31 (4); 39.

[15] Lazarow A, Wells LJ, Carpenter AM, Hegre OD, Leonard RJ, Mc Evoy RC. Banying Lecture 1973: Islet differentiation, organ culture and transplantation. Diabetes 1973; 22: 877 - 912.

[16] Brown J, Danilovs JA, Clarck WR, Mullen YS. Fetal pancreas as a donor organ. World J Surg 1984; 8: 152 - 157.

[17] Brown J, Clarck WR, Molnar IG, Mullen YS. Fetal pancreas transplantation for reversal of streptozotocin-induced diabetes in rats. Diabetes 1976; 25: 56 - 64.

[18] Lafferty KJ, Hao L, Babcock SK, Spees E. Is there a future for fetal pancreas transplantation? Transplant Proc 1989; 21: 2611-2613.

[19] Farkas GY, Karacsonyi S. Clinical transplantation of fetal human pancreatic islets. Biomed Biochim Acta 1985; 44: 155 -159.

[20] Robertson RP, Lafferty KJ, Haug CE, Weil III R. Effect of human fetal pancreas transplantation on secretion of C-peptide and glucose tolerance in type I diabetics. Transplantation Proc 1987; xix: 2354-2356.

[21] Brown J, Heininger D, Kuret J, Mullen Y. Islet cells grow after transplantation of fetal pancreas and control of diabetes. Diabetes 1981; 30: 9-13.

[22] Mullen YS, Clark WR, Molnar IG, Brown J. Complete reversal of experimental diabetes mellitus in rats by a single fetal pancreas. Science 1977; 195: 68.

[23] Spence RK, Perloff LJ, Barker CF. Resident's corner. Fetal pancreas in treatment of experimental diabetes in rats. Current surgery 1981; 38: 138-140.

[24] Mc Evoy, Hegre OD. Syngeneic transplantation of the fetal rat pancreas. Diabetes 1979; 28: 141 – 146.

[25] Brown, J., Danilovs J.A., Clark W.R., Mullen Y.S. (1984). Fetal pancreas as donor organ. World J Surg 1984; 8: 152 – 157.

[26] Du Toit DF, Muller C, Page B, Mattyssen J, Lyners R. Immunosuppression With Cyclosporin A in Combination With Mycophenolate Mofetil Suppresses Rejection of Allogeneic Fetal Rat Pancreatic Allografts. Transplant Proc 1998; 30 (8): 4092 – 4093.

[27] Watkins PJ. Diabetes and its management. Oxford: Blackwell Scientific Publications; 1993.

Chapter 3

TRANSPLANT IMMUNOLOGY

Rejection

Rejection, an unknown phenomenen in early attempts at pancreas transplantation, severely affected pancreas allograft survival and thwarted the efforts of early researchers. The introduction of modern immunosuppressants especially cyclosporine, discovered by Borrel in 1976, offered hope of arresting the rejection process and in more consistent survival of allografts[1,2].

Our knowledge and understanding of the immune system and the mechanism of allograft rejection has greatly increased and continues to expand rapidly on molecular and genetic levels[3]. The discovery of monoclonal antibodies targeted against various cell-surface molecules has enabled us to identify the various cellular role players and cytokines involved in the rejection process.

The usual immunological rejection process against foreign antigens, eg. allograft is mediated by T-cells which, on encountering the foreign tissue, destroy these cells either by direct cytotoxicity or by the release of cytokines. The target molecules recognised by these T-cells are mainly the major histocompatibility complexes (MHC) antigens, but can, in addition be targeted at the minor histocompatibility antigens[3]. These minor compatibility antigens are peptides, thought to be derived from polymorphic molecules other than the MHC complex[3].

Major Histocompatibility Complex (MHC)

The major histocompatibility complex (MHC) molecules act as cellular identification markers which bind peptide antigens both intracellularly (presented on MHC class one) and

extracellularly (presented on MHC class two), allowing cells of the immune system (CD8 and CD4 T-lymphocytes respectively) to monitor normal cells[3]. There are distinct pathways in which peptides become associated with either the MHC class one or class two molecules.

In class one molecules, endogenous peptides are produced by proteosomes from intracellular proteins and transported by peptide transporters to the endoplasmic reticulum. In the endoplasmic reticulum the class one molecule is formed and peptide fragments of between 8 – 10 amino acids are incorporated into the peptide-binding groove. The peptides are bound in an extended β-structure with the amino and carboxy ends tightly bound to the opposite ends of the groove.

Extracellular proteins, ingested into the cell by endocytosis, are degraded by lysosomes in an acidic endosomal compartment where the class two molecule is bound to it. Class two binding peptides are longer, between 12 and 28 amino acids and more variable in size. The binding groove of the class two molecule is open at both ends and the peptides are not as tightly bound. The bound peptides often bulge out of the open ends and rest on top of the binding groove[3].

The cells of the immune systems are carefully selected and are constantly reminded of what is self, as identified by these MHC antigens. Major histocompatibility complex antigens are encoded on at least three separate loci on chromosome 6 in humans (termed HLA A, -B, -C for class 1 and HLA DP, -DQ, -DR for class 2). In the mouse the MHC is encoded on chromosome 17 (termed H2D, -L, -R for class 1 and H2A, -E, -J for class 2). Each MHC has between 6 – 40 or more alleles resulting in the extreme polymorphism found in each individual. This creates the high likelihood of histoincompatibilities between unrelated individuals [4].

MHC class one molecules consisting of a polymorphic α-chain associated with a nonpolymorphic β2 microglobulin chain are present on all nucleated cells but are particularly abundant on lymphoid cells and vascular endothelium. Major histocompatibility complexes class two molecules consist of covalently linked dimers of α- and β-chains. Major histocompatibility complexes class two molecules are expressed on antigen presenting cells (dendritic and Langerhans cells along with B cells, macrophages and vascular endothelium) and activated T-cells. Various immunological mediators called cytokines modulate MHC expression. Cytokines like interferon α, -β and -γ along with TNF α and -β are potent inducers of MHC class 1 antigen expression. Induction of increased MHC class 2 expression is more restricted, with interferon-γ being a potent inducer of class 2 expression.

Increased MHC expression increases the immunogenic potential of an allograft explaining the link between virus infection and graft rejection while the inhibition of cytokines by cyclosporin, an IL-2 inhibitor, generally lowers the antigenicity of the graft.

Recognition of alloantigens

T-cells are selected in the thymus to weakly bind the MHC with their T-cell receptors. The T-cell subtypes CD4 and CD8 react with MHC class 2 and MHC class 1 antigens respectively[5]. T-cells are extremely sensitive to any structural differences associated with the MHC and have been shown to be able to detect and reject a skin allograft differing with only 1 – 3 amino acids in either the class 1 or 2 MHC antigens[4]. The introduction of MHC mismatched allogeneic antigens, mismatched by either their major- and/or minor histocompatibilty antigens, results in a rapid and potent immune response.

The minor histocompatibilty antigens are antigens derived from proteins particular to an individual (eg mitochondrial proteins) resulting in peptides/antigens derived of different polymorphic molecules to the MHC but show similar donor to recipient differences[3,6]. The response is enhanced by the different ways in which these antigens are presented to the effector cells of the host.

Direct recognition of alloantigens

Direct recognition of the allogeneic MHC molecules as self-plus-X, by the great diversity of host T-cell receptors is highly likely and leads to a rapid activation of cytotoxic T-cells. This early acute response may involve as many as 5 to 10% of the total peripheral T-cell pool caused by the high density of MHC alloantigens on the graft. Some T-cells are peptide specific while others are MHC specific but able to bind the various peptides associated with that MHC molecule and some T-cells bind directly to the empty MHC molecules of the donor cells[3, 5 ,7].

Indirect recognition of alloantigens

If the host does not react directly against the MHC antigens of the graft two other mechanisms could induce destruction of the graft. Firstly, the antigen presenting cells within the graft could present its own peptides in association with its MHC inducing a cytotoxic T-cells response or the hosts antigen presenting cells could present the foreign graft peptides in association with its MHC thus initiating a response against the foreign peptide[5,8,9,10]. The destruction of the grafts presented to the effector cells in this manner would be mediated by lymphokines[3].

T-Cell activation molecules

The antigen specificity of the T-cell receptor is provided by clonal restriction and consists of two chains, an α- and a β-chain linked by disulfide bridges. The CD3 molecule is noncovalently bound to the T-cell receptor (TCR), the cytoplasmic domains of the CD3 molecules are much longer than the TCR's α- and β-chains and thus provides a link between the TCR and the intracellular signaling pathway. The invariant chains of the CD3 molecule contain a common domain that couples these proteins to the intracellular protein kinases[3]. Two protein kinases known to react with the antigen recognition activation motifs are *lck* and *fyn*, both members of the *Src* family.

The CD4 and CD8 molecules are bound to the cytoplasmic protein tyrosine kinase *lck* though a cysteine-containing motif shared by their cytoplasmic domains. CD4 and CD8 coreceptors therefore bring *lck* into the proximity of the TCR complex, where it acts on phosphorylated tyrosines within the antigen recognition activation motifs of the CD3 and ζ-chains (intracytoplasmic proteins associated with the TCR) early in the signal transduction pathway. Later the SH2 domain of the CD4-associated *lck* is bound to the stimulated TCR complex by tyrosine phosphorylated residues in the TCR complex. The binding of CD4/*lck* to the TCR complex increases the avidity and effectivity of signaling of the TCR/MHC interaction [3].

Binding of the TCR induces the tyrosine phosphorylation of a host of cytoplasmic and membrane proteins. Phosphorylation of phospholipase C-γ1 increases its activity and causes the cleavage of phophatidyl inositol biphosphate, which results in the generation of the second messengers, inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate induces a sustained rise in intracellular calcium while diacylglycerol activates protein kinase C. These two signals

synergise to induce and activate DNA-binding factors needed for IL-2 gene transcription. The rise in intracellular calcium activates calcineurin, a calcium-dependant serine/threonine phosphatase that modifies the cytoplasmic component of nuclear factor of the activated T-cells resulting in it translating to the nucleus. There it combines with newly formed *Fra-1* and *JunB* proteins, induced by the diacylglycerol activated protein kinase C pathway, to form a functional nuclear factor of activated T-cell complex, a positively acting transcription factor on the IL 2 enhancer gene[3]. Tyrosine phosphorylation of *vav* leads to the activation of the *ras* signal transduction pathway. The oncogene, ras is a guanine triphosphate-binding protein that interacts directly with the serine-threonine kinase, *RAF-1*, which regulates the activity of the kinase cascade including Mek and mitogen-activated protein (Map) kinase, leading to cell proliferation and differentiation by inducing *c-fos* and *c-jun* transcription factors. Expression of an activated form of *ras* potentiates IL-2 promoter activity whereas the negative form of *ras* inhibits IL-2 promotor activity. T-cell stimulation also causes the nuclear factor of κB transcription factor to translocate to the nucleus which also regulates the stability of cytokine mRNA by the induction of RNA-binding factors [3].

T-cell costimulation pathway

Effective stimulation of naïve T-cells by antigen presenting cells (APC's) requires a secondary non-antigen specific signal, provided by costimulation molecules. This is a safety mechanism in addition to positive and negative selection in the thymus and prevents naïve T-cells from reacting against self-antigens. Failure to provide the costimulatory signal during antigen presentation by an antigen-presenting cell to a receptive naïve T-cell results in anergy. Many costimulatory molecules have been identified including the various intercellular adhesion molecules ICAM-1,

ICAM-2 and ICAM-3 and lymphocyte function associated molecule 3 (LFA-3) on the surface of APC's binding to LFA-1 and LFA-2 on the T-cells does enhance T-cells proliferation but blockage of these molecules does not induce anergy.

The major costimulatory signal appears to be the CD28 molecule binding to the B7 family present on the APC's. Engagement of the TCR in the absence of CD28/B7 costimulation results in failure to induce an immune response resulting in anergy and preventing transplant rejection [3] [11] [12]. In humans CD28 is expressed on 95% of resting CD4 cells and on 50% of resting CD8 cells in the peripheral blood.

CD28 is structurally homologous to cytolytic T-lymphocyte antigen 4 (CTLA-4) which is expressed on activated T-cells which delivers an negative secondary signal, further modulating T-cells response. CTLA-4 knock-out mice die after 4 weeks due to an uncontrolled accumulation of activated T-lymphocytes[3]. Both these costimulatory molecules are deemed important in preventing transplant rejection[11] [13, 14].

CD4 T-cells

Mature T-cells express either CD4 or CD8 molecules that bind the MHC class 2 and MHC class 1 respectively. CD4 binds to the β2 segment of the MHC class 2 molecule whereas CD8 binds to the α3 segment of the class 1. CD4 lymphocytes are sub classified further by their lymphokine production. T-helper 1 (Th-1) cells are CD4 lymphocytes responsible for the delayed hypersensitivity type reactions and secrete interferon-γ and TNF-α. T-helper 2 (Th-2) lymphocytes provide B cell help and secrete IL-4, IL-5, IL-6 and IL-10[4].

Differentiation of effector T-cells following antigen stimulation results from a regulated cascade of gene activation including the proto-oncogenes *c-myc* and *c-fos*, which are transcribed minutes after T-cell activation. These early activation gene products along with ongoing signal transduction give rise to the next wave of gene activation including the transcription of the IL-2 and IL-2 receptor gene. T-cell proliferation is driven by IL-2 secretion, acting on T-cells expressing the IL-2 receptor thereafter the other cytokines like IL-3, IL-4, IL-5, IL-6 and IFN-γ are also produced. After the initial burst of cytokine secretion T-cells divide and differentiate under the influence of IL-2 and IL-4[14,15].

Th-1 and Th-2

Activated CD4 cells are further subclassified according to the lymphokines they produce. Th-1 cells mainly secrete IL-2, TNF-α and IFNγ and Th-2 cells mainly secrete IL-4, IL-5, IL-6 and IL-10. These Th-1 and Th-2 cells originate from the same precursor, Th-0 cells, and cross regulate each other, e.g. IFNγ inhibits Th-2 and IL-4 and IL-10 suppresses Th-1[16]. TGF-β seems to be a cytokine that initiates the transformation of Th-0 to Th-1 and suppresses both IL-4 and IL-10 but not IL-2 thus encouraging Th-1 clonal expansion. Whether the transformation from Th-0 to either Th-1 or Th-2 is permanent and whether these clones can be reverted back to a Th-0 or switched between Th-1 and Th-2 is a contentious issue, but studies have shown that IFN-γ secretion could be transiently induced in human allergen specific Th-2 clones by IL-12. The suppressive effect of continuous IFN-γ secretion in Th-1 or IL-4, and to a lesser extent IL-10, in Th-2 clones are the most likely mechanisms by which the cytokines secretory pattern of either Th-1 or Th-2 clones is maintained [15].

CD4 T-cells and rejection

Rejected grafts typically contain CD4 cells in which the Th-1 phenotype predominates, IFNγ being the key cytokine in the rejection process as it activates macrophages, endothelial cells and increases MHC expression along with transporter proteins associated with antigen presentation[3]. The role of IL-10 is still controversial having both an immunesuppressing and immunestimulating effect. IL-10 is seen in grafts during the induction and maintenance phases of tolerated grafts but is absent in rejecting grafts. Inhibition of IL-10 with specific antibodies leads to rejection of the graft[3].

CD8 T-cells and rejection

CD8 cytotoxic T-cells are able to kill allograft cells by two different mechanisms. Firstly CD8 cells secrete cytolytic granules that contain a variety of cytolytic proteins including perforin, a complement like molecule, and granzymes, a type of serine protease. Perforin polymerizes the cell membrane of target cells resulting in osmotic lysis of the cells. Granzyme induces apoptosis by deregulating the normal cell cycle process. Granzyme B and perforin are expressed highly in grafts undergoing acute rejection[3]. Although CD8 cells can induce apoptosis in the target cells via the FAS/FAS ligand pathway it is probably more important in the controlling of lymphocyte proliferation than graft destruction[3]. Inhibition of either CD4 or CD8 cells in mixed MHC class 1 and 2 incompatibility allografts prolongs graft survival, suggesting some synergy between the subsets in the graft rejection process[4].

BIBLIOGRAPHY

[1] Borrel JF, Feurer C, Gubler HU, Stähelin H. Diabetological effects of cyclosporine A: a new antilymphocytic agent. Agent Actions 1976; 6: 468.

[2] Du Toit DF, Heydenrych JJ. Siklosporien: 'n nuwe en belowende immuunonderdrukkende middel. Geneeskunde 1986; Maart: 31 - 35.

[3] Pattison JM, Krensky AM. New insights into mechanisms of allograft rejection. Am J Med Sci. 1997; 313(5): 257-63.

[4] Colvin RB. Cellular and molecular mechanisms of allograft rejection. Annu Rev Med 1990; 41: 361-375.

[5] Konig R. Interactions between MHC molecules and co-receptors of the TCR. Curr Opin Immunol 2002; 14 (1): 75 - 83.

[6] Nevala WK, Paul C, Wettstein PJ. Immunodominant minor histocompatibility antigent peptides recognized by cytolytic T-lymphocytes primed by indirect presentation. Transplantation 1998; 65: 559-569.

[7] Game DS, Lechler RI. Pathways of allorecognition: implication for transplantation tolerance. Transpl Immunol 2002; 10 (2-3): 101 - 108.

[8] Harris PE, Cortesini R, Suciu-Foca N. Indirect allorecognition in solid organ transplantation. Rev Immunogenet 1999; 1(3): 297 - 308.

[9] Sayegh MH, Carpenter CB. Role of indirect allorecognition in allograft rejection. Int Rev Immunol 1996; 13 (3): 221 - 9.

[10] Bradley JA. Indirect T-cell recognition in allograft rejection. Int Rev Immunol 1996; 13 (3): 245 - 255.

[11] Alegre M, Fallarino F, Zhou P, Frauwirth K, Thistlethwaite J, Newell K, Gajewski T, Bluestone J. Transplantation and the CD28/CTLA4/B7 pathway. Transplant Proc 2001; 33(1-2): 209-11.

[12] Kishimoto K, Dong V M, Sayegh MH. The role of costimulatory molecules as targets for new immunosuppressives in transplantation. Curr Opinion Urology 2000; 10: 57-62.

[13] Gudmundsdottir H, Turka LA. T-cell costimulatory blockade: new therapies for transplant rejection. J Am Soc Nephrol 1999; 10 (6): 1356 - 1365.

[14] Divate SA. Acute renal allograft rejection: process in understanding cellular and molecular mechanisms. J Postgrad Med 2000; 46 (4): 293 - 6.

[15] Pawelec G, Rehbein A, Scholtz E, Friccius H, Pohla H. Cytokine modulation of Th-1/ Th-2 phenotype differentiation in directly allo responsive $CD4^+$ human T-cells. Transplantation 1996; 62: 1095-1101.

[16] Fitzpatrick DR, Kelso A. Independent regulation of cytokine genes in T-cells transplantation. The paradox in the Paradigm. Transplantation. 1998; 65(1): 1-5.

Chapter 4

IMMUNESUPPRESSION AND TOLERANCE

Tolerance

In 1953 Medawar and his colleagues found that by exposure of the immune system to alloantigen during foetal development or the neonatal period induced tolerance to this antigen provided the presence of the antigen was maintained[1]. It is clear that various mechanisms are involved in the induction and maintenance of tolerance. Tolerance can be divided into central and peripheral tolerance.

Central (intra-thymic) tolerance

It is generally accepted that the thymus is the site of central tolerance induction. The thymus provides the unique microenvironment for the proliferation of T-cell precursors. Immature thymocytes proliferate in the cortex at an astonishing rate of $5x10^7$ cells/day of which only about $1x10^6$ mature cells emigrate from the thymus into the periphery each day[2]. This means that approximately 98% of thymocytes generated in the thymus each day die by either positive or negative selection. Early double positive $CD4^+CD8^+$ thymocytes that express TCRs that cannot recognise and bind to self MHC complexes, expressed on the thymic epithelial cells, fail positive selection and are eliminated. Self-reactive thymocytes that bind with high avidity to the MHC complexes of thymic epithelial cells and dendritic cells are selectively (negative selection) removed by apoptosis[2,3,4]. These cells are eliminated from the pool even if costimulated by B7/CD28. Doubly positive thymocytes that pass both the positive and negative selection process lose either CD4 or CD8 and increase their expression of TCR[2]. These nearly mature thymocytes then encounter bone marrow derived dendritic cells in the cortico-medullary and medullary regions of the thymus [2]. Dendritic cells are thought to be the most potent cells in eliminating

self-reactive T-cells during intrathymic development[5]. The presence of donor derived dendritic cells following the establishment of donor microchimerisms and subsequent graft acceptance could be due to this interaction[6]. The role of the thymus in the induction of self-tolerance has been undisputed for years and is clearly demonstrated in thymectomised newly born mice which develop a wide-spread autoimmune syndrome affecting various organs[7]. Similarly ablation of the thymic medullary epithelial cells by cyclosporine resulted in organ-specific autoreactivity[7]. However, thymic selection only occurs against intra-thymic antigens. Originally described by Waksman more that 30 years ago, injection of self- or allo-antigen into the thymus leads to a therapeutic induction of specific tolerance[7,8,9]. Transplanting allografts into early neonates results in the expression of alloantigen on the surface of thymic epithelial cells. This induces *in vivo* tolerance to the allograft[3]. Besides some proof that activated peripheral T-cells might re-enter the thymus medulla, it is accepted that extra-thymic tissue antigens are ignored by the thymus[3,8].

T-cells develop in the thymus, and as a result of random rearrangements of their antigen receptor genes, they mature into thymocytes expressing both CD4 and CD8 on their surface (DP thymocytes). In order to proceed to the single CD4 or CD8 positive (SP) mature thymocyte, the T-cell receptor must interact with self-peptides (Ag) bound in the clefts of the major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC). A high-avidity (and therefore potentially autoimmune) interaction with self-Ag/MHC will lead to central thymic removal, mainly by clonal deletion, whereas a low-avidity interaction leads to positive selection and export into the periphery as a naive T-cell. Immune responses in the periphery (spleen, lymph nodes, Peyers patches, etc) are also subject to control mechanisms that include clonal deletion and anergy as well as regulation and suppression. These mechanisms are

important both to maintain tolerance to self-antigens that were not expressed in the thymus during negative selection and might be recognized by the emerging naive T-cells, as well as to limit responses to foreign antigens by primed T-cells once the invading pathogen has been eliminated by the immune response. The aim in using therapeutic agents such as monoclonal antibodies that block CD4 and CD8 molecules *in vivo* is to harness and amplify these natural tolerance and regulatory mechanisms to control responses to foreign tissues following transplantation of self-tissues in autoimmune disease (from Waldmann *et al.* 1998[10]).

Peripheral (post-thymic) tolerance

Despite extensive negative selection (elimination) in the thymus, self-reactive T-cells are inadvertently transported to the periphery[8]. Peripheral tolerance is the mechanism that ensures a second line of control against immune reaction to self-antigens in the periphery. The peripheral component eliminates, inactivates, downregulates (suppresses) or polarises (Th-1 - Th-2) autoreactive clones of T-cells to extrathymic expressed self-antigens[8]. Several non-exclusive mechanisms are thought to regulate peripheral tolerance[3].

In older animals with a competent peripheral immune system, central tolerance alone is not sufficient to induce tolerance and the emphasis shifts to the induction of peripheral T-cell tolerance. The induction of peripheral tolerance has become the most readily exploited modality in the context of organ transplantation[6].

Deletion of the peripheral immunologically competent cells can be achieved in various ways. Whole body irradiation and the use of antilymphocyte antibodies (ALS) cause indiscriminate destruction of all lymphocytes leading to severe immune deficiency rather than tolerance to the

allogeneic cells. Clonal deletion of reactive cells to allogeneic antigens can be effected by Fas-mediated induction of apoptosis[4]. The Sertoli cell of the testis has been implicated in the maintenance of the immune privilege found in the testis. These cells secrete CD95L (Fas-ligand) and it is postulated that when T-cells become activated they express CD95 (Fas) on their cell surface. Binding of the surface CD95 by the ligand leads to programmed cell death (apoptosis) of the activated cell, thus resulting in clonal deletion of those cells[11]. Selawry and Cameron attempted co-transplantation of testis tissue and allogeneic pancreas islets in 1993, but they found that prolonged survival of the graft was only seen in animals that received a course of CsA post-transplantation. This could have been due to the cell purification and separation techniques employed resulting in a low yield of viable Sertoli cells which were unable to provide sufficient Fas ligand immediately post-transplantation[12,13]. Korrbutt improved the harvesting technique by culturing testicular cells for 48 hours, which resulted in the formation of cell aggregates found to consist of Sertoli cells. These aggregates were then be transplanted together with the pancreas islets under the kidney capsule. Results showed that provided sufficient testicular cells were co-transplanted long-term survival up to 105 days was possible[13]. The growth factors that are secreted by Sertoli cells e.g. IGF-1, TGF-β and laminin are further benefits to pancreas graft survival and development [13].

Immunesuppressive agents

Many immunesuppressive agents and therapies have been employed in transplantation and the list of immunesuppressive agents is growing rapidly. Currently autoimmune diseases, allergies and transplant rejection therapy, treated with anti-inflammatory and immunesuppressive drugs are the third biggest demand, behind cancer and cardiovascular disease, on health services in the western world[10]. Immunesuppressive agents/therapies are not specific to the antigen and have a general suppressive effect on the whole immune response thus reducing the immune potency of the patient. Clinically a balance has to be found between sufficient immunesupression to prevent graft rejection and the complications (infection and malignancy) caused by too much immunesuppression[14].

Intense immunesuppression leading to a high degree of immunodeficiency can be achieved by indiscriminate depletion of lymphocytes. This strategy is often employed during rejection episodes and induction therapy during the initial days of transplantation. Polyclonal antibodies like antilymphocyte serum (ALS) and ATG - a rabbit anti-human T-lymphocyte immune globulin and monoclonal antibodies aimed at the CD3 molecule e.g. OKT3 are very effective. Both these antibodies induce a rapid depletion of the T-cells which is dose dependent leaving the individual either partially or completely immunologically incompetent or highly vulnerable to opportunistic infections and tumours. A similar result can be achieved by irradiation of the lymphoid organs[14].

A less drastic approach to immunesuppression is to modulate or suspend the immune response following allogeneic transplantation. The immune response offers several opportunities for

intervention. Antigen recognition by the T-cell induces a cascade of events, which is required to induce an effective immune response to the antigen.

Regulatory cells

A large body of evidence is emerging that clearly shows that tolerance does not equal the absence of alloreactivity. CTL assays of tolerised animals have shown the presence of alloreactive cytotoxic T-cells. This demonstrates the complexity of the underlying tolerogenic mechanisms[3].

Two models for the origin of regulatory T-cells have been proposed. In the so-called "choices" model it is postulated that naive T-cells stimulated by antigen under conditions of inadequate help or costimulation become tolerant regulatory T-cells. These cells are in an alternate state of activation and consequently would require a continued source of antigen to maintain the appropriate state of activation. Akdis, Yssel and Weiner who have presented evidence that T-regulatory cells are differentiated and formed from naïve T-cells in the periphery on encountering antigens present in high concentrations support this hypothesis[15]. The second model is referred to as the "lineage" model proposes that regulatory T-cells are produced in the thymus which are committed only to a suppressive function on encountering the appropriate antigenic stimulation[16]. A group of prominent immunologists including Stevens and Masson support this theory suggesting that T-regulatory cells leave the thymus as a distinct subset of mature T-cells[15].

In both cases, the generation of T regulatory cells is an active process, which requires the continued presence of the antigen, and it would appear that these suggested mechanisms are connected[15]. Maintenance of T-regulatory cells is known to be highly dependent on a continuous supply of antigen. Withdrawal of antigen results in a rapid decline in the suppressive capabilities of T-regulatory cells[16].

T-regulatory cells have been implicated in the induction of active peripheral tolerance and the sequential induction of "infectious" tolerance in subsequent non-tolerised recipients[17,18,19]. The first notion that CD4 T-cells could be mediating suppression in transplantation was made by Hall *et al.* in 1985 following findings that a short period of CsA treatment prolonged allograft survival in rats. This was at a time when it was accepted that T- suppressor cells were within the CD8 subset[16]. The existence of CD4 T-cells that could suppress graft rejection in mice following a short course of non-depleting anti-mouse CD4 and CD8, was shown in 1993 by Qin and co-workers[20,16]. Since then the demonstration of CD4 suppressor cell involvement in graft tolerance has been shown in a wide range of rodent transplantation models[16].

The generation of regulatory cells to relevant self-antigens is emerging as one of the most important tolerogenic mechanisms in the periphery.

Certainly the thymus is critical for the establishment of regulatory cells and tolerance by anti-CD4/donor specific antigen therapies[15]. Countinho and co-workers proposed a thymus selection model, which proposes that thymocytes binding a ligand with high avidity would enter a T-reg cell pool[8,21].

Introduction of intraocular antigens into the eye, an immuneprivileged site, induces rapid antigen specific $CD4^+CD25^+$ T regulatory thymocytes inducted by eye-derived APCs[8]. Beschorner *et al.* showed the presence of donor-origin DCs in the corticomedulla of CsA treated rats injected intravenously with lymphopoietic cells[22]. $CD4^+CD25^+$ regulatory thymocyte release to the periphery only occurred once the CsA therapy was stopped. This suggests that there is an association between the entrance of circulating DCs into the thymus and the establishment of acquired thymic tolerance by $CD4^+CD25^+$ regulatory thymocytes[8].

Evidence is also presented which shows that the introduction of antigen intravenously and orally induces antigen-specific $CD4^+CD25^+$ T-reg cells from naïve CD4 precursors in the periphery. It appears that cytokines/ APCs interaction plays an essential role in the establishment of peripheral T-regulatory cells. Stimulation of T-cells with immature dendritic cells (DC) leads to the development of T-regulatory cells. IL-10 has been shown to inhibit DC maturation and could be central to the establishment and maintenance of peripheral T-reg cells[23,24,25,26].

Regulatory T-cells (Tr-1 cells) have been shown to express CD4, CD25, $CD45RB^{low}$, and constitute 5 – 10% of the peripheral $CD4^+$ cells in normal mice[26,27,28,29]. Elimination of these cells induces various autoimmune diseases[27]. Studies have shown these CD4 cells do not proliferate on antigen stimulation but suppress proliferation of other antigen reactive T-cells. These cells are able to suppress rejection by both naive and primed CD4 and CD8 T-cells[16].

Activation and expansion of self-reactive T-cells, that have escaped thymic clonal deletion is actively suppressed in the periphery by naturally occurring $CD4^+$ regulatory T-cells, the majority of which constitutively express CD25 (IL-2 receptor α-chain). *Foxp3* a common gene, which encodes a forkhead-winged-helix transcription factor designated Scurfin expression, is

predominantly expressed by and restricted to the $CD25^+CD4^+$ population in both the thymus and periphery[30]. *Foxp3* may be a master regulatory gene for cell-lineage commitment or developmental differentiation of regulatory T-cells in the thymus and the periphery. Therefore *Foxp3* might be a more specific marker than currently used cell-surface molecules (such as CD25, CD45RB, CTLA-4, and GITR), which are unable to completely discriminate between regulatory T-cells and activated, effector, or memory T-cells[30].

The cytokine profile of these cells is still contentious with some authors reporting that these cells do not secrete cytokines while some evidence has been shown that these cells transcribe for IL-4, IL-10 and TGF-β[15,26,29].

Discrepancies are also noted in the mechanism of action in these $CD4^+CD25^+$ cells. In vitro studies generally show that the suppressive effect of these $CD4^+CD25^+$ cells are caused by cell-cell interactions mainly with the APCs resulting in suppression of IL-2 production. The *in vivo* scenario is very different to that of the in vitro here IL-10 and TGF-β production by these regulatory cells have been shown to be the most likely suppressive mechanism[26,31,32,33]. Anti-TGF-β1 mAb has been shown to reverse $CD4^+CD25^+$ suppression in a dose specific manner[28]. $CD4^+CD25^+$ cells express surface membrane bound TGF-β both in its latent and active configuration on their cell surfaces. Enhanced levels of TGF-β receptor type II are present on the surface of these cells[28]. An unexpected finding that triggering of the TCR of responder cells induces upregulation of TGF-β receptor type II has provided a possible explanation of how T-reg cells interact with responder/effector cells[28]. Direct TGF-β receptor/ TGF-β/ TGF-β receptor binding between the T-reg cell and a T-effector cell mediates a suppressive biochemical pathway. Binding of the TGF-β receptor II by TGF-β phosphorylates TGF-β receptor I which

propagates a signal by phosphorylation of cytosolic proteins including Smad proteins (Smad2/3) which then translocates to the nucleus where they modulate specific genes including genes that promote IL-2 production[28].

$CD4^+CD25^+$ cells also express the T-lymphocyte-associated antigen-4 (CTLA-4), a negative costimulatory signal following T-cell activation. Acute rejection is associated with an upregulation of CD28/CTLA-4/B7 molecules on peripheral blood T- and B-cells, clearly indicating the importance of this co-stimulatory pathway in the rejection process[29,34]. The precise mechanism whereby CTLA-4 signaling contributes to suppression of effector cells is still unknown Binding of CTLA-4 is thought to induce TGF-β production[26,28]. The preferential binding of CTLA-4 to CD80 and CD86 thus preventing CD28 costimualtory signaling is another possibility[26]. Blockage of the CD28/B7 pathway by CTLA-4 has been shown to lead to indefinite survival of islet, cardiac and renal allografts[35,36].

The role of B-cells as APCs in liver transplantation is of particular interest as high levels of serum IL-10 post-transplantation hinders the ability of macrophages and dendritic cells to stimulated Th-1 effector cells but not Th-2. B7 molecules on monocytes remain at basal levels during acute rejection episodes in liver transplants while B-cells express high levels of B7. In non-rejecting liver transplants B-cells are mostly B7 negative (resting B-cells) indicating that at least in liver rejection B-cells are the APCs that modulate rejection through CD28/CTLA-4/B7 costimulation[34].

There is enough evidence to show that TCR and CD28 transduce early stimulatory signals in the T-cell immune response and that CTLA-4, which is expressed later, inhibits the response

possible by reducing IL-2 secretion or apoptosis[34]. CTLA-4 has a higher affinity for B7 than CD28 but produces a weaker signal. Therefore, alloantigens presented to T-cells by APCs that have low levels of B7 will result in prefertial binding of CTLA-4 resulting in a tolerogenic response. Antigen presentation by APCs with high levels of B7 results in stronger CD28 signaling which overrides the weaker CTLA-4 signal thereby inducing T-cell activation and rejection[34]. The CD28/CTLA-4/B7 costimulation scenario is further complicated by the different stimulatory signals elicited by the different B7 molecules. Blockade of B7-1 and CTLA-4 promotes and accelerates rejection, while blockage of B7-2 prolonged graft survival[37,38].

CD4+/CD25+ cells have been shown to be particularly responsive to inflammatory cytokines especially those engaging the CCR4 and CCR8 receptors. This could indicate that these cells actively home in on sites of inflammation[39].

T-cell anergy

Effective ligation of the TCR by antigen/MHC complex molecules along with an appropriate co-stimulatory signal (B7/CD28) induces activation of the reactive cell resulting in cytokine secretion and clonal expansion of the reactive cells. Ineffective binding of the TCR-antigen/MHC complex and/or failure to activate a costimulatory signal, such as B7/CD28, results in an attenuated activation signal, which is insufficient for an effective immune response, leads to specific T-cell anergy[3,4,10]. *In vivo* antigen-specific naive T-cells require the collaborative interaction between specialised APCs and the correct environment i.e. cytokines and appropriate co-stimulatory signals. If the number of antigen-specific naive cells is too low or has insufficient avidity to form a stable cluster around the APC, the correct environment is not created and these cells revert to a tolerance/anergic state. These anergic cells compete for antigen and if enough

are present, a state of general tolerance against a specific antigen is induced. This process is analogous to the process whereby therapeutic antibodies like anti-CD4, both depleting and non-depleting, induce tolerance. The antibody either depletes the antigen-reactive cells to such an extent that the remaining cells cannot produce a stable cluster/environment for an effective response or with an non-depleting antibody, blockade of activation molecules like CD4 reduces the avidity of these anti-reactive cells which then default to an anergic state. Waldmann refers to this as the civil service state because the minimum requirement of the tolerant T-cells (analogous to government officials) is the ability to passively interfere with the attempts of naive T-cells (analogous with public interest groups) to get a response started[10].

Anergic cells classically produce little IL-2, IL-4 and IFN-γ. The downregulation of IL-2 secretion seems to be one of the major pathways of inducting anergy. Anergic cells produce significant amounts of IL-10 and TGF-β, both regulatory cytokines[6]. IL-10 has been shown to induce an antigen-specific anergy in CD4 cells. These cells fail to proliferate or produce cytokines in response to antigen stimulation[32]. Another characteristic of anergy is that effector T-cell function can be restored by treatment with IL-2[40]. Targeting the ERK-1/2 signalling pathway has been shown to induce alloantigen specific anergy in human T-cells. Following engagement of the TCR, various kinases are activated, including extra cellular-related kinase (ERK), these inturn transactivate various gene products necessary for T-cell function. Krieger *et al.* has demonstrated that blocking ERK downregulates CD25 thus suppressing IL-2 dependent proliferation of T-cells[41]. An interesting finding by Krieger *et al.* was that addition of CsA to ERK-pathway blockade promoted this ERK-induced alloantibody T-cell anergy[41].

Negative selection of T-cells in the periphery

Elimination of antigen specific T-cells occurs in the periphery. A similar system to the thymus selection of high avidity to antigens is present in the periphery. T-cells bearing high affinity TCRs to specific antigens are eliminated by apoptosis while T-cells with a weaker affinity to the specific antigen clonally proliferate. This prevents excessive T-cell clonal expansion and is a self-protection mechanism[3]. Candidate sites for peripheral clonal deletion include the gut, bone marrow and the liver. Van Pel *et al.* showed that thymecomised mice are still able to develop donor-specific tolerance after sublethal conditioning consisting of a single dose of anti T-cell monoclonal antibody and low dose total body irradiation. The authors were able to demonstrate the presence of donor T-cells and donor APCs in the GALT of the small intestine thereby concluding that the gut was the primary organ supplying the microenvironment for clonal deletion of alloreactive cells, in euthymic and thymecomised mice[42].

Microchimerism and tolerance

Billingham, Brent, and Medawar in 1953 were the first to show that it was possible to induce chimerism-associated neonatal tolerance deliberately[1]. This discovery escalated over the next 15 years to the first successful bone marrow transplantations in humans in 1968[43].

Donor cells and genetic material can usually be detected within a recipient following organ transplantation. This phenomenon is referred to as peripheral (micro)chimersim, which in some cases can be long lasting. Donor material has been detected in the periphery of recipients up to 29 years after renal transplantation[44,45]. Starzl *et al.* in 1992 were the first to show that that long-surviving organ transplant recipients had persistent microchimerisms. They then put forward the hypothesis that organ transplantation induced peripheral microchimerisms which are associated

with long-term acceptance of allografts and that this process plays an active role in the induction and maintenance of unresponsiveness[43,45,46]. Passenger leukocytes i.e. leukocytes present within the transplanted organ, especially immature dendritic cells migrate to recipient's lymphoid tissues, especially the spleen, where these cells interact with recipient $CD4^+$ cells thereby eliciting an immunological response[45]. This process contributes greatly to the immunogenicity of the grafts[45,47]. Chimerisms can be introduced to establish tolerance. In practice this can be achieved by either donor transfusion or donor bone marrow transplantation. The establishment of a mixed lymphohaematopoietic chimerisms resulting in a low but persistant levels of donor-derived dendritic cells which are capable of providing a persistent source of antigen in the thymus and thereby effecting negative selection and establishing donor specific tolerance[45,46,48].

CsA and FK506

Immunosuppressants like cyclosporine (CsA), tacrolimus (FK506) bind to cyclophilin and FK-binding protein respectively to form an intra-cytoplasmic active complex, which in turn binds to and inhibits calcineurin. Inhibition of calcineurin prevents activation of cytokine promotor genes (e.g. IL-2, IFN-γ, GM-CSF, TNF-α, IL-4 and CD40L) and therefore prevents cytokine-induced proliferation[49]. Both cyclosporine and tacrolimus are highly effective in graft maintenance with tacrolimus being more potent. Clinical trails comparing FK506 to CsA found that liver and renal patients on FK506 had fewer acute rejection episodes and less refractory and chronic rejection. More patients became hyperglycaemic requiring insulin following FK506 but fewer patients had elevated cholesterol[49]. Nephrotoxicity is a potential side effect of both FK506 and CsA, which is probably due to vasospasm in the kidney, induced by calcineurin inhibition. Infectious

complications were higher in the FK506 groups but could be due to the higher level of immunosuppression[49].

Sirlolimus

Sirolimus (Rapamycin) is a antibiotic similar to FK506 binding to the same intracellular binding protein (FKBP). Unlike FK506, the sirolimus – FKBP complex does not inhibit calcineurin but it binds to and inhibits the autophosphorylation of the target of rapamycin, a protein with a kinase domain[49]. Inhibition of the target of rapamycin interrupts the signaling pathway between the cytokine receptors and the cell cycling arresting cells at the G1- to S-phase transition during their cell cycle. Studies combining sirolimus with CsA have show a reduction of acute rejection but an outbreak of *Pneumocystis carinii* during the trial suggests excessive immunodeficiency induced by the combination[49].

Mycophenolate mofetil (MMF)

Mycophenolate mofetil (MMF) and its active agent mycophenolic acid (MPA) which inhibits inosine monophophate dehydrogenase (IMPDH) the rate limiting enzyme in *de novo* purine biosynthesis, therefore interrupting the *de novo* purine synthesis pathway, which is required by activated lymphocytes for cell division. Most other cells are able to use the salvage pathway and proliferate normally. MMF combined with CsA has shown similar efficacy compared to CsA and azathioprine but MMF does not have the same marrow toxicity[49]. MMF does have gastrointestinal toxicity, caused by the high levels of glucuronide in the bile. The efficacy of MMF is further enhanced if combined with FK506 which appears to alters the MPA metabolism

increasing its half-life. MMF has also been shown to suppress glycosylation and adhesion molecules in human monocytes[49].

Immunosuppression and tolerance

In contrast to original beliefs that cyclosporine does not induce tolerance, and in fact counteracts tolerance induction, evidence is slowly emerging that under certain circumstances cyclosporine could help induce tolerance. Hall *et al.* showed in 1985 that they could achieve prolonged cardiac allograft survival in rats after a brief period of treatment with CsA[16,50] [combination of a short course of CsA (7x daily doses) with DST (1x weekly) four weeks before transplantation of cardiac grafts into MHC class I disparate rats]. In this research, Yang *et al.* made a strong case that the major role players in the induction of tolerance in these rat models were, not as expected the CD4 T-cells but appeared to be mediated in an allospecific manner by B-cells[51]. Studies on rat cardiac allografts have shown that combining DST with a short course of CsA significantly suppressed alloantibody formation[51]. Tolerance was broken by co-infusion of normal B-cells and CD4 T-cells which resulted in high levels of allospecific cytotoxic antibodies[51]. The graft unresponsiveness was not always permanent and often grafts were lost in time[51]. CsA alone has been shown to have little effect on B-cell proliferation but has been shown by several groups to prevent apoptosis of immature T- and B-cells while CsA has been shown to directly mediate apoptosis in mature lymphocytes[51].

Antibodies used for immunosuppression

Polyclonal antibodies like anti-leukocyte serum (ALS), anti-thymocyte globulin (ATG) deplete the peripheral immune system of T-cells, by activating the complement system following binding of the relevant antibody to the cell. Depletion of the T-cells is dose dependant leaving the individual partially or completely immune deficient. This form of treatment is used as induction therapy and is also used for rescue therapy during a rejection episode. The indiscriminate action of polyclonal therapy is due to the nature of polyclonal antibodies i.e. a cocktail of antibodies against various individual antigens introduced and occurring naturally in the animals in which the antibodies are being raised.

Monoclonal antibodies on the other hand can be raised to a single antigen and are extremely specific. Monoclonal antibodies can also be either depleting i.e. inducing cytolysis by complement activation or non-depleting where the antibodies bind the surface antigens without activation of complement. OKT3 an anti-CD3 monoclonal antibody was the first monoclonal antibody approved for transplant immunosuppression. OKT3 clears T-cells (CD3 positive cells) from the circulation within minutes, leaving the patient severely immune compromised. Some T-cells do however eventually return to the blood but initially these cells do not express surface CD3 molecules, and since CD3 interacts with the T-cell receptor these cells have greatly reduced function[52].

$CD4^+$ T-cells are known to play a central role in most immune responses. CD4 cells recognize exogenously derived antigen, presented by antigen presenting cells (APC) and they are the cells that drive the activation of the effector cells against these antigens. Targeting the CD4 molecule with various monoclonal antibodies in order to manipulate the response has shown promising

results in various experimental animal models. Depleting CD4 monoclonal antibodies has been shown to induce permanent acceptance of grafts. In 1986 the Waldmann and Wofsy groups showed that a short course of depleting CD4 mAb induced long-term tolerance to foreign immunoglobulins in rats and mice respectively. This finding was further supported by the findings of Fathman that the depleting anti CD4 mAb L3T4 suppressed the response to sperm whale myoglobulin in mice[10]. One of the first rat anti-mouse CD4 mAb GK1.5 (isotype IgG2b) antibodies used *in vivo,* shown to be highly cytolytic in the presence of complement, blocked all MHC class II antigen specific functions including cytolysis, proliferation and release of cytokines [53]. Depletion of the CD4 cells is dose dependent but even high doses can only remove approximately 90-95% of the CD4 cells from the peripheral blood, lymph nodes and spleen. The remaining 5-10% of antibody resistant cells are cells expressing low levels of CD4 and are thought to be memory cells[53]. The thymus protects the CD4 thymocytes from cytolysis and even high doses of GK1.5 mAb have little effect on the thymocyte populations. Following a maximal dose of 5 mg/kg GK1.5 the CD4 cells returned to pretreatment levels at around 100 days.

In rats, similar results were achieved using a mouse anti-rat CD4 mAb OX-38. A high dose of 9-13mg/kg induces long-term depletion of CD4 cells, with only 50 – 60% of the original CD4 cells being present at 80 days post-treatment. These rats were however still immunocompetent with the ability to reject third party grafts and to mount mixed lymphocyte and cytotoxic T-cell responses[53].

The recovery of the peripheral CD4 cells, following treatment with depleting mAb, due to replenishment from precursors re-establishes immunocompetence but in an altered form[53]. Burkhardt (1989) compared the efficacy of the depleting CD4 mAb GK1.5 to a nondepleting

CD4 mAb H129.19, a rat anti-mouse CD4 mAb isotype IgG2a (shown to be a poor inducer of complement-mediated lysis), in preventing foetal pancreas allograft rejection in fully MHC mismatched mice[54]. Examination of the splenic T-cell populations, following anti CD4 treatment, showed that GK1.5 (anti-L3T4a)[55] had removed almost all the $CD4^+$ (<1%) cells from the spleen while >95% of the $CD4^+$ cells that were present, demonstrated the presence of rat IgG on their surfaces[54]. They found that both the depleting and non-depleting mAb prevented graft rejection at 14 days but rejection occurred at 28 days.

The cells infiltrating the graft were almost exclusively CD8 positive cells. They concluded that the CD8 cells were able to reject both MHC I and MHC II mismatched allografts but at a slower rate but that the addition of CsA to both the mAb had a synergistic effect, improving graft survival and suppressing graft infiltration by CD8 cells[54]. Although Burkhardt was disappointed in the results he made a valid comment that long-term acceptance of isolated adult islets had been achieved in other strain combinations[54].

Waldmann showed that it was possible to induce tolerance in an allogeneic marrow transplants, under the cover of nondepleting CD4 and CD8 mAbs, in animals with multiple minor MHC mismatches[16]. These mice then became chimeric and accepted skin grafts of the donor. The induction of tolerance in fully MHC mismatched animals could not be achieved by CD4 and CD8 mAb alone but by further immunosuppression by irradiation (600 rads) or other myeloablation using the alkylating agent dimethylmyeleran (DMM)[10].

The differences in efficacy of CD4 mAb in different animal models was shown by Ilano (1989) in a study using anti CD4 mAbs OX35 and OX38 for transplantation of PVG vascularized neonatal heart allografts in low responding DA (RTIa) and high responding Wistar Furth (W/F)

rats[56]. The low responder DA rats had indefinite survival of all their grafts while the high responding W/F rats showed prolonged graft survival compared to the controls, they had all rejected their grafts at 42 days. Comparison of the MLC proliferation of the T-cell subsets using irradiated PVG spleen cells as stimulators on $CD4^+$ and $CD8^+$ enriched cell populations showed a marked difference in the alloreactivity in these strains[56].

In the high responding W/F model $CD8^+$ cells proliferated following stimulation while the $CD8^+$ cells in the low responding DA rats showed no increase in cell numbers. CD4 proliferation, following stimulation, was high in both the high and Low responding models. It would therefore appear that in high responding animals $CD8^+$ cells proliferated upon antigen stimulation without the presence of CD4 cells, by producing exogenous cytokines thus overcoming the need for CD4 help[56].

The lack of responsiveness seen in the low responders (DA) is not due to a defect in their immune response. Rejection can be restored by infusion of as little as $1x10^6$ naïve $CD4^+$ cells while the infusion of $CD8^+$ cells could not reverse the unresponsiveness. Proliferation of the $CD8^+$ cell could be induced in culture by cytokine manipulation. Addition of anti-CD8 (OX8) mAb to the anti-CD4 therapy prevented the induction of unresponsiveness[56].

Manipulation of the early CD4 positive T-cells by anti-CD4 mAbs, following antigen exposure, to induce a more tolerant Th-2 response has become a popular method of prolonging graft survival in various animal models. W3/25, a mouse anti rat anti-CD4 mAb, isotype IgG_1 binding to domain 1 of the CD4 molcule[57], was shown to inhibit the mixed lymphocyte response (MLR) of PVG/c ($RT1^c$) lymphnode as responding cells stimulated with HO.B2($RT1^U$) spleen cells as well as other strain combinations including AO vs DA and PVG/c vs DA. A concentration of 25

ng/ml gave maximal inhibition of the MLR for allogeneic stimulation but could not inhibit the MLR response following T-cell mitogenic stimulation with concanavalin A (con A) or phytohaemagglutinin (PHA) even if the concentration of the W3/25 was increased to 2.5 ug/ml, a 100 times that required to maximally inhibit alloantigen stimulated MLR's[58,59]. Inhibition of these W3/25 positive cells (CD4+ lymphocytes) was specific without killing them. A control mAb W3/13 (CD43), did not affect the MLR of these cells[58].

W3/25 was shown to be effective in the treatment of experimentally induced allergic encephalomyelitis (EAE) in Lewis rats. A single 1.5 mg i.p. injection of W3/25 was administered at the first sign of encephalomyelitis i.e. limp tail, hind leg weakness or hind leg paralysis, occurring normally at day 12 – 13 after immunization with guinea pig MBP. Following W3/25 administration affected rats recovered in a day or two compared to 5 – 6 days in the control animals[60]. A further study on EAE, Mannie looked at the possible mechanisms and effect of W3/25 therapy in this EAE model. Using reverse transcription polymerase chain reaction (RT-PCR) he could show that the W3/25 profoundly reduced the IL-2/IL-4 cDNA ratio and although the IL-4 mRNA was reduced the IL-2 mRNA was totally inhibited following allogen stimulation but not mitogen stimulation[59].

In vitro studies did however show that although IL-2 production might be slightly reduced, an increase of IL-2 was detectable after 48 hours in a 1° MLC before proliferation was detected and continued to increase as proliferation increased in a similar manner to that seen in uninhibited controls[57]. Cells activated in the presence of W3/25 also displayed normal levels of IL-2 receptor. What was very apparent was that IFN-γ production was completely inhibited in the W3/25 treated 1° MLC. This inhibition of IFN-γ synthesis by W3/25 had a dramatic effect on

the synthesis of IL-4. Quantitative RT-PCR of 1° MLCs showed similar message levels between the W3/25 treated and control groups for IL-4, IL-10 and IL13. IL-4 levels showed an dramatic 300 times increase in IL-4 synthesis compared to the untreated control groups in a 2° MLC[57]. Supplementation of the W3/25 treated MLC with IFNγ and IL-2 induced a 50% increase in proliferation while addition of these cytokines to the untreated control cultures had no effect. IL 13 showed a similar effect with IL13 levels increased 100-fold that of the control cultures in the 2° MLC. In contrast the IL-10 synthesis was not increased with similar levels between the W3/25 and non-treated control cultures[57].

Experimentation to confirm the increased production of IL-4 in the 2° MLC of the cells treated with W3/25 in the 1° MLC, the ability of IL-4 or IL13, present in high levels in the supernatant of the W3/25 treated 2° MLC, to upregulate MHC class II expression on B-cells was assessed. Both 24-hour and 48-hour supernatant significantly upregulated MHC class II expression on the B-cells, which could be blocked by the addition of OX81, which is a neutralizing mAb to rat IL-4[57]. Enhanced IL-4 production in the 2 ° MLC is most likely caused by the inhibition of IFN-γ since the addition of IFN-γ to the 1° MLC of W3/25 treated cultures blocked the subsequent increase of IL-4 production in the 2° MLC. An interesting finding was that IFN-γ synthesis was not inhibited in the 2° MLC showing that the inhibition of IFN-γ seen in the 1° MLR could be reversed[57].

IFN-γ has been shown to promote Th-1 clones over Th-2 clones in mice[57,61]. Proliferation of Th-1 cells also requires that the peptide to which a response is generated is present in abundance and binds with great affinity to the MHC class II molecules of the APC. Therefore compared to the Th-2 cells, Th-1 cells would require a greater number of high affinity peptide-MHC

interactions to be activated. Anti-CD4 mAb binding of CD4 interferes with MHC-CD4 costimulation[57,62], providing an attenuated signal. Due to the attenuated signal it is quite conceivable that only Th-2 cells proliferate if antigen is introduced under the cover of an anti-CD4 mAb and more specifically W3/25[57]. Proliferation without CD4 costimulation, inhibited by the antibody, and the high levels of IL-4 and IL-13 secreted by these CD4 positive cells in a 2^{o} MLC, under cover of W3/25 in the 1^{o} MLC would support such a theory[57].

This would suggest that W3/25 and very likely other anti-CD4 mAbs interfere with CD4 costimulation of T-cells following antigen recognition, differentially promoting Th-2 proliferation and cytokine secretion of especially IL-4 and the inhibition of Th-1 cytokines especially IFN-γ and TGF-β production[57,59,63,64].

Induction of tolerance

Ondera using the nondepleting mAb RIB5/2 in his rat cardiac allograft model and Waldmann's group, using anti-CD4 and anti-CD8 mAb in MHC minor mismatched skin allografts in thymectomized mice showed that the tolerance achieved, was infectious and that naïve recipients could be tolerised by splenocytes of tolerant animals[10,65]. Ondera showed that the infectious tolerance could be abrogated by depletion of CD4+ cells with a depleting anti-CD4 mAb (OX36) while depletion of CD8+ cells with anti-CD8 mAb (OX8) had no effect, proving that CD4+ cells were required to maintain tolerance[10,65]. These $CD4^{+}$ regulatory T-cells, generated in response to alloantigen in the presence of anti-CD4 mAb, may well represent putative immunoregulatory Th-2-like cells which migrate to the spleen and may be sequestered at the graft site in tolerant animals[65].

These cells, on alloantigen stimulation, are associated with increased expression of IL-4 and IL-10 mRNA in the grafts of both primary and secondary recipients[65]. The ability of anti-CD4 mAb to preferentially promote Th-2 cells could be explained by the fact that the ligation of the surface CD4 molecule by the CD4 mAb activates protein tyrosine kinase $p56^{lck}$, which is associated with the cytoplasmic portion of the CD4 molecule. Since Th-2 cells use protein tyrosine kinase for signal transduction, activation of $p56^{lck}$ by cross linking of the CD4 molecule with anti-CD4 mAb, increases levels of cAMP in these Th-2 lymphocytes, which in turn could provide a negative signal to inhibit Th-1 activation[66].

Alloantigen and CD4 antibody induction of tolerance

Functional tolerance by introducing donor specific antigen (peripheral blood or splenocytes) under the cover of anti-CD4 mAbs has been demonstrated in various rat and mouse models[29,67,68,69,70]. The most likely mechanism of tolerance induction by anti-CD4/Donor specific antigen is by regulatory cells, both during the induction phase and the maintenance of tolerance[19,29,65]. This anti-CD4 induced immune regulation effective suppresses naïve and activated CD4 and CD8 cells in an antigen specific manner[19]. Furthermore the acquired anti-CD4 tolerance can be transferred from the recipient into a secondary recipient, which clearly indicates the role of regulatory cells[19]. Woods and the Oxford transplant immunology group have demonstrated in a rodent model that if DST is given in combination with anti-CD4 mAb therapy 28 days prior to transplantation these rats will accept the grafts without any further immunosuppression. This crucial period is thought to be required for the clonal development of specific regulatory cells[29,70].

Alloantibodies

Anti-CD4 mAb therapy with RIB5/2 in the cardiac model prevented the class switch of alloantibodies from IgM to IgG and was able to suppress IgG alloantibody production indefinitely in that model[65]. The prevention of this class switch may be critical for long-term acceptance of allografts as the deposition of intragraft IgG is a classical feature of chronic rejection[65]. Immunoglobulin class switching is induced by complex T- and B-cell interactions in which the cytokines they produce especially IFN-γ plays a major role[65]. Furthermore, the production of antiidiotypic antibodies in response to the injected monoclonal antibodies is severely suppressed by anti-CD4 monoclonal antibodies showing little or no humoral reactivity to the injected antibody, provided sufficient antibody is given[65,71].

Th-1 and Th-2 and graft tolerance.

It has been hypothesised that deflection of the allogeneic immune response from a Th-1 like response, which is associated with acute graft rejection, to a Th-2-like response may help to establish and maintain graft acceptance or even tolerance. The activity of so-called suppressor cells which represent anergic cells which tolerise or actively suppress other non-tolerant cells may be explained by the Th-1/Th-2 paradigm[64]. Pawelec *et al.* showed differentiation of human Th-0 T-cell phenotyes, to either Th-1 or Th-2, following alloantigen stimulation via the direct pathway could be manipulated in vitro by the neutralization or addition of certain cytokines. Brief exposures of IL-4 or IL-10 diverted the response to that of a Th-2 while TGF-β favored the development of a Th-1 phenotype[64].

The generally accepted principle that Th-1 cytokines promote graft rejection while Th-2 cytokine favors acceptance or functional tolerance of allografts has been questioned by some researchers. Using a depleting mouse anti-CD8 mAb, in a mouse model, Chan *et al.* showed cytokines (IL-2, IFN-γ, IL-4, IL-5 and IL-10) expressed in their rejecting cardiac allografts to be of a mixed Th-1/Th-2 type. In comparison the cytokine profiles of the unmodified rejection groups consisted of only Th-1 cytokines with IL-2 and IFN-γ being present and no IL-4 and IL-5 present. In the anti-CD8 treated group, the intragraft infiltrate consisted of macrophages, lymphocytes and a significant amount of eosinophils, which were not present in the unmodified rejection groups. Eosinophils, know to be responsive to Th-2 cytokines and IL-5 in particular which they have been shown to be the effector cells in the rejection process of these grafts[72].

A similar scenario exists in transgenic CD95L expressing islets allografts, which instead of being immune priviliged and causing apoptosis of graft infiltrating effector cells, (the grafts) were rapidly rejected by neutrophils[73,74,75]. Despite these and other experiments Th-2 cytokines, and especially IL-4 has been shown to be essential for the establishment of neonatal tolerance[76]. *In vivo* introduction of anti-IL-4 in neonates prevents the induction of tolerance by IL-4 secreting tolerogenic T-cells[77].

Donor specific antigens and transfusions.

Similarly, the introduction of foreign alloantigens into the developing immune system of neonatal mice induces specific tolerance in the mouse. These tolerised mice will accept allografts from the specific donors indefinitely but reject third party grafts[1].

In adults donor-specific transfusions have been shown to have a modulating effect on graft rejection and has been show to prolong cardiac and renal allograft survival which is associated with an decrease in mRNA for IL-2 and IFN-γ and an persistent production of IL-4 and IL-10 mRNA [78]. While this is very effective in selective strain combinations, unresponsiveness is not always maintained and most grafts are eventually rejected[68]. The Nuffield group of Sir Peter Morris and Kathryn Wood has actively researched combination of donor-specific antigen and anti-CD4 mAb to induce donor unresponsiveness. Fully H-2 and minor mismatched adult mice combinations received two injections of YTS 191.1 (50 ug) on days –28 and –27 prior to transplantation. On day –27 they also injected 0.25 ml donor specific whole blood. No further treatment was given and the animals transplanted. Following this protocol most cardiac grafts survived more than 100 days but the authors acknowledge that they could not induce the same graft acceptance in other fully mismatched allogeneic strain combinations[68]. Using a similar depleting anti-CD4 mAb YTA 3.1.2 Bushell of the same group irradiated the donor blood prior to transfusion to see whether the graft acceptance was due to microchimerisms from stem cells within the donor blood. Interestingly, if the DST was treated with a single high dose of irradiation (2000 rads) rejection followed but if he gave three doses of the irradiated blood DST the tolerogenic effect was restored. From this he concluded that the graft acceptance, following this protocol, was most likely due to the persistence of alloantigen during the 3 days in which the anti-CD4 mAb had its effect and not due to the establishment of a microchimerism[79]. Woods *et al.* describes the effect of anti-CD4 and alloantigen therapy as a perturbment of the proximal signalling events following TCR/CD3 ligation, resulting in reduced tyrosine phosphorylation of Zap-70 and LAT and reduced association of tyrosine-phosphorylated LAT with *lck*. This ultimately results in severely reduced proliferation of the responding $CD4^+$ T-cells. The

signalling profile of the anti-CD4-treated cells resembled that of anergic T-cells. This could be a result of a common mechanism involving perturbation in the formation of the central supramolecular activation cluster of the immunological synapse by impaired recruitment of CD4 and CD28, thereby resulting in severely reduced lck activation[80].

DST in the clinical setting

Medawar in 1946 first reported accelerated graft rejection of skin transplants in rabbits that had received prior blood transfusions[81]. The immunomodulating effect of allogeneic blood transfusions was first revealed by a positive association between kidney graft survival and the number of allogeneic transfusions received by the recipient[82]. In a randomised clinical study Opelz *et al.* found that despite the use of new potent immunosuppression the beneficial effect of blood transfusions on kidney graft survival is still observed[83].

Several immulogical mechanisms have been proposed that may be involved with the tolerogenic/immunomodulatory effect of donor transfusion. These include clonal deletion, clonal anergy, introduction of anti-idiotypic antibodies and regulatory cells. Transfusions are also thought to induce a Th-2 type response rather than a Th-1 response. The cytokines produced by Th-2 cells include IL-4, IL-10 and TGF-β which are able to downregulate cytotoxic T-cells[82,84,85]. The shift to a Th-2 response will occur if the antigen presenting cells in the blood are unable to provide the proper co-stimulatory signals to T-cells[82]. Blood transfusions introduce allogeneic MHC II bearing APCs, which can directly activate CD4 recipient helper cells. However the ex vivo storage of blood affects the effectivity of the APC and in particular their ability to provide costimulatory signals essential for T-cell activation[86].

The fate of donor lymphocytes post transfusion is not well known. The establishment of a donor cell microchimersim, demonstrating tolerance between donor and recipient has been demonstrated for more than a year in multiple transfused trauma patients[86].

Evidence is emerging that soluble factors in the blood transfusion product may play an important role. Supernatant of blood products contain several soluble molecules with immunomodulatory properties. These include soluble HLA class I and soluble FAS ligand molecules. These molecules are able to block the reactivity of alloantigen specific T-cells by inducing apoptosis of the potentially reactive cells[82].

One of the main reasons for not employing pretransplant transfusions in the clinical setting is the inadvertent introduction of alloantibody, which renders the patients unsuitable for transplantation. However, the DST-induced sensitisation might in fact be less harmful than initially perceived. In fact, the presence of alloantibody after DST infusion could identify recipients with elevated risk of rejection requiring aggressive anti-rejection therapy. The non-antibody producers will benefit from the DST modulating effect on T-cell-mediated rejection[87].

BIBLIOGRAPHY

[1] Billingham RE, Brent L, Medawar PB. Actively aquired tolerance of foreign cells. Nature 1953; 172: 603.

[2] Janeway CA, Travers P. Immunobiology: The immune system in health and disease. Oxford: Blackwell Scientific Publishers; 1994.

[3] Arnold B. Levels of peripheral T-cell tolerance. Transpl Immunol 2002; 10: 109 – 114.

[4] Aradhye S, Turka LA. Will Tolerance Become a Clinical Reality? Am J Med Sci 1997; 313 (5): 310 -314.

[5] Janeway CA, Goodnow CC, Medzhitov R. Immunological tolerance: danger – antigen on the premises. Curr Biology 1996; 6 (5): 519 – 522.

[6] Lechler R, Bluestone JA. Transplantation tolerance putting the pieces together. Curr Opin Immunol 1997; 9(5): 631-3.

[7] Kyewski B, Derbinski J, Gotter J, Klein L. Promiscuous gene expression and central T-cell tolerance: more than meets the eye. Trends in Immunology 2002; 23 (7): 364 – 371.

[8] Goldschneider I, Cone RE. A central role for peripheral dendritic cells in the induction of acquired thymic tolerance. Trends in Immunology 2003; 24 (2): 77 – 81.

[9] Stadlbauer THW, Schaub M, Magee CC, Kupiec-Weglinski JW, Sayegh MH. Intrathymic immunomodulation in sensitized rat recipients of cardiac allografts: requirements for allorecognition pathways. J Heart Lung Transplant 2000; 19 (6): 566 – 575.

[10] Waldmann H, Cobbold S. How do monoclonal antibodies induce tolerance? A role for infectious tolerance? Annu Rev Immunol 1998; 16: 619-44.

[11] Vaux DL. Immunology. Ways around rejection. Nature 1995; 377(6550): 576-7

[12] Selawry HP, Cameron DF. Sertoli cell-enriched fractions in successful isleT-cell transplantation. Cell Transplant 1993; 2(2): 123-9.

[13] Korbutt GS, Elliott JF, Rajotte RV. Cotransplantation of allogeneic islets with allogeneic testicular cell aggregates allows long-term graft survival without systemic immunosuppression. Diabetes 1997; 46(2): 317-22

[14] Halloran, P.F. Immunosuppresive agents in clinical trials in transplantation. Am J Med Sci 1997; 313: 283-288.

[15] Groux H. An overview of regulatory T-cells. Microbes and Infection 2001; 3: 883 – 889.

[16] Waldmann H. Regulating the immune response to transplants: a role for CD4+ regulatory cells? Immunity 2001; 14: 399 – 406.

[17] Karim M, Bushell AR, Wood KJ. Regulatory T-cells in Transplantation. Curr opinion in Immunol 2002 14: 584 - 591

[18] Ondera K, Chandraker A, Volk HD, Lehmann M, Korom S, Stadlbauer THW, Kato K, Kasai S, Sayegh MH, Kupiec-Weglinski JW. Role of regulatory T-cells in the "infectious" tolerance pathway in transplant recipients. Transplant Proc 1998; 30: 13 – 15.

[19] Cobbold S, Waldmann H. Infectious tolerance. Current Biology 1998; 10: 518 – 524.

[20] Qin S, Cobbold SP, Pope H, Elliott J, Kioussis D, Davies J, Waldmann H. "Infectious" transplantation tolerance. Science 1993; 259(5097): 974-7

[21] Modigliani Y, Coutinho A, Pereira P, Le Douarin N, Thomas-Vaslin V, Burlen-Defranoux O, Salaun J, Bandeira A. Establishment of tissue-specific tolerance is driven by regulatory T-cells selected by thymic epithelium. Eur J Immunol 1996; 26(8):1807-15

[22] Beschorner WE, Yao X, Divic J. Recruitment of semiallogeneic dendritic cells to the thymus during post-cyclosporine thymic regeneration. Transplantation 1995; 60(11): 1326-30.

[23] Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4(+) T-cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med 2000; 192(9): 1213-22

[24] Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T-cell function in humans after injection of immature dendritic cells. J Exp Med 2001; 193(2): 233-8

[25] Powrie F, Maloy KJ. Immunology. Regulating the regulators. Science 2003; 299(5609): 1030 - 1.

[26] Read S, Powrie F. CD4+ regulatory T-cells. Curr opinion in Immunology 2001; 13: 644 – 649.

[27] Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T-cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155(3): 1151-64.

[28] Chen W, Wahl SM. TGF-β: the missing link in CD4+CD25+ regulatory T-cell-mediated immunosuppression. Cytokine and Growth 2003; 14: 85 – 89.

[29] Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T-cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. J Immunol 2002; 168(3):1080-6.

[30] Hori S, Nomura T, Sakaguchi S. Control of regulatory T-cell development by the transcription factor Foxp3. Science 2003; 299(5609):1057-61

[31] Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T-cells. Exp Med 1996; 184(1): 19 - 29.

[32] Groux H. An overview of regulatory T-cells. Microbes Infect 2001; 3(11): 883-9.

[33] Zelenika D, Adams E, Humm S, Graca L, Thompson S, Cobbold SP, Waldmann H. Zregulatory T-cells overexpress a subset of Th-2 gene transcripts. J Immunol 2002; 168: 1069 – 1079.

[34] Minguela A, Martin L, Torio A, Muro M, Garcia-Alonso AM, Moya-Quiles MR, Sanchez-Bueno F, Parrilla P, Alvarez-Lopez MR. CD28/CTLA-4 and CD80/CD86 costimulatory molecules are mainly involved in acceptance or rejection of human liver transplant. Human Immunology 2000; 61: 658 – 669.

[35] Rastellini C, Salam A, Kuddus R, Aitouche A, Braun M, Leach r, Peach R, Fung JJ, Starzl TE, Rao AS. Mechanisms underlying the developemnt of T-cell tolerance following interruption of signalling at the CD28/B7 and CD40/gp39 interface. Transplant Proc 1999; 31: 845.

[36] Perico N, Remuzzi G. Prevention of transplant rejection – curent treatment guidelines and future developments. Drugs 1997; 54 (4); 534 – 570.

[37] Yamada A, Kishimoto K, Dong VM, Sho M, Salama AD, Anosova NG, Benichou G, Mandelbrot DA, Sharpe AH, Turka LA, Auchincloss H Jr, Sayegh MH. CD28-independent costimulation of T-cells in alloimmune responses. J Immunol 2001;167(1):140-6

[38] Guerder S, Carding SR, Flavell RA. B7 costimulation is necessary for the activation of the lytic function in cytotoxic T lymphocyte precursors. J Immunol 1995; 155(11): 5167-74

[39] Karim M, Bushell AR, Wood KJ. Regulatory T-cells in transplantation. Current opinion in Immunology 2002; 14: 584 – 591.

[40] Ng WF, Baker RJ, Hernandez M, Chaudhry A, Lechlol RI. The role of T-cell anergy in the maintenance of donor-specific hyporesponsiveness in renal transplant recipients. Transplant Proc 2001; 33: 154 –155.

[41] Krieger P-M, Säermann GA, Böhmig GA, Mühlbacher F, Zlabinger GJl. Targeting the ERK-1/2 signalling pathway results in alloantigen-specific anergy in human T-cells. Transplant Proc 2002; 34: 1403 – 1404.

[42] van Pel M, Hilbrands L, Smits D, van Breugel DW, van Eck M, Boog CJ. Permanent acceptance of both cardiac and skin allografts using a mild conditioning regimen for the induction of stable mixed chimerism in mice. Transpl Immunol 2003; 11(1): 57-63.

[43] Starzl TE. History of clinical transplantation. World J Surg 2000; 24(7): 759-82.

[44] Starzl TE, Demetris AJ, Trucco M, Zeevi A, Ramos H, Terasaki P, Rudert WA, Kocova M, Ricordi C, Ildstad S. Chimerism and donor-specific nonreactivity 27 to 29 years after kidney allotransplantation. Transplantation. 1993; 55(6): 1272 - 7.

[45] Wood K, Sachs DH. Chimerism and Transplantation Tolerance. Immunology Today 1996; 17 (12): 584 - 587.

[46] Starzl TE, Demetris AJ, Trucco M, Ramos H, Zeevi A, Rudert WA, Kocova M, Ricordi C, Ildstad S, Murase N Systemic chimerism in human female recipients of male livers. Lancet. 1992; 340(8824): 876 - 7.

[47] Lechler RI, Batchelor JR. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. J Exp Med. 1982; 155(1): 31 - 41.

[48] Tomita Y, Khan A, Sykes M. Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantation in mice conditioned with a nonmyeloablative regimen. J Immunol. 1994; 153(3): 1087 - 98.

[49] Halloran, PF. Immunosuppresive agents in clinical trials in transplantation. Am J Med Sci 1997; 313: 283-288.

[50] Hall BM, Jelbart ME, Gurley KE, Dorsch SE. Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. Mediation of specific suppression by T-helper/inducer cells. J Exp Med 1985; 162(5): 1683-94.

[51] Yang C-P, Sittu E, Bell EB. Specific B-cell tolerance is induced by cyclosporine A plus donor-specific pretreatment: prolonged survival of MHC class I disparate cardiac allogreafts. J Immunol 2000; 164: 2427 – 2432.

[52] Colvin R.B.. Cellular and molecular mechanisms of allograft rejection. Annu Rev Med 1990; 41: 361-375.

[53] Shizuru JA, Alters SE, Fathman CG. Anti-CD4 monoclonal antibodies in therapy: creation of nonclassical tolerance in the adult. Immunol Rev 1992; 129:105-30.

[54] Burkhardt K, Charlton B, Mandel TE. An increase in the survival of murine H-2-mismatched cultured fetal pancreas allografts using depleting or nondepleting anti-CD4 monoclonal antibodies, and a further increase with the addition of cyclosporine. Transplantation 1989; 47(5): 771-5.

[55] Dialynas DP, Wilde DB, Marrack P, Pierres A, Wall KA, Havran W, Otten G, Loken MR, Pierres M, Kappler J, et al.. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T-cell clones appears to correlate primarily with class II MHC antigen-reactivity. Immunol Rev 1983; 74: 29 - 56.

[56] Ilano AL, Mcconnell MV, Gurley KE, Spinelli A, Pearce NW, Hall BM. Cellular basis of allograft rejection in vivo. V. Examination of the mechanisms responsible for the differing efficacy of monoclonal antibody to CD4+ T-cell subsets in low- and high-responder rat strains. J Immunol 1989; 143(9): 2828-36.

[57] Stumbles P, Mason D. Activation of CD 4+ T-cells in the presence of a non-depleting monoclonal antibody to CD 4 induces a Th2- type response in vitro. J Exp Med 1995; 182: 5-13

[58] Webb M., Mason DW, Williams A.F. Inhibition of mixed lymphocyte response by monoclonal antibody specific for rat T-lymphocyte subset. Nature 1979; 282: 841.

[59] Mannie MD, Morrison-Plummer J, Mc Connel TJ. Differentiation of Encephalitogenic T-cells Confers Resistance to an Inhibitory Anti-CD4 Monoclonal Antibody. J Immunol 1993; 151: 7293.

[60] Brostoff SW, Mason DW. Experimental allergic encephalomyelitis: successful treatment in vivo with a monoclonal antibody that recognizes T-helper cells. J Immunol 1984; 133(4): 1938-42.

[61] Gajewski TF, Joyce J, Fitch FW. Antiproliferative effect of IFN-gamma in immune regulation. III. Differential selection of TH-1 and TH-2 murine helper T-lymphocyte clones using recombinant IL-2 and recombinant IFN-gamma. J Immunol 1989; 143(1):15-22.

[62] Van Den Broek MF, Van De Langerijt LGM, Van Brugggen MCJ, Billingham MEJ, Van Den Berg WB. Treatment of rats with monoclonal anti-CD 4 induces Longterm resistance to Streptococal cell wall-induced arthritis. Eur J Immunol 1992; (22): 57-61.

[63] Pelegrí C, Morante MP, Castellote C, Castell M., Franch A. Administration of a non-depleting anti-CD 4 monoclonal antibody (W3/25) prevents adjuvant arthritis even upon re-challenge: parallel adminisration of a depleting anti-CD 8 monoclonal antibody (OX8) does not modify the effect of W3/25. Cellular Immunology 1995; (165): 177-182.

[64] Pawelec G, Rehbein A, Scholtz E, Friccius H, Pohla H. Cytokine modulation of Th-1/ Th-2 phenotype differentiation in directly allo responsive CD4+ human T-cells. Transplantation 1996; (62): 1095-1101.

[65] Ondera K, Hancock W, Graser E, Lehmann M, Sayegh MH, Strom TB, Volk H-D, Kupiec-Weglinski JW. Type 2 helper T-cell type cytokines and the development of 'infectious" tolerance in rat cardiac allograft recipients. J Immunol 1997; 158: 1572-1581.

[66] Munoz E, Zubiaga AM, Merrow M, Sauter NP, Huber BT. Cholera toxin discriminates between T-helper 1 and 2 cells in T-cell receptor-mediated activation: role of cAMP in T-cell proliferation. J Exp Med 1990; 172(1):95-103

[67] Motoyama K, Lehmann M, Flye MW. Analysis of donor-specific unresponsiveness to rat allografts by nondepleting anti-CD4 monoclonal antibody (RIB 5/2) plus donor antigen. Transplant Proc 1999; 31(1-2): 1237-8.

[68] Pearson TC, Madsen JC, Larsen CP, Morris PJ, Wood KJ. Introduction of transplantation tolerance in adults using donor antigen and anti-CD4 monoclonal antibody. Transplantation 1992; 54: 475 – 483.

[69] Arima T, Lehman M, Flye MW. Induction of donor specific transplantation tolerance to cardiac allografts following treatment with non-depleting (RIB 5/2) or depleting (OX-38) anti-CD4 mAb plus intrathymic or intravenous donor alloantigen. Transplantation 1997; 63: 284-292.

[70] Saitovitch D, Bushell A, Mabbs DW, Morris PJ, Wood KJ. Kinetics of Induction of Transplantation Tolerance with a Nondepleting Anti-CD4 Monoclonal Antibody and Donor-specific Transfusion Before Transplantation. Transplantation. 1996; 61: 1642.

[71] Lehmann M, Sternkopf F, Metz F, Brock J, Döcke W-D, Plantikow A, Kuttler B, Hahn H-J, Ringel B, Volk H-D. Induction of Longterm survival of rat skin allografts by novel highly efficient anti-CD 4 monoclonal antibody. Transplantation 1992; 54: 959-962.

[72] Chan SY, Debruyne LA, Goodman RE, Eichwald EJ, Bishop DK. In vivo depletion of CD8+ T-cells results in Th-2 cytokine production and alternate mechanisms of allograft rejection. Transplantation 1995; 59(8): 1155-61.

[73] Kang SM, Schneider DM, Lin Z, Hanahan D, Dichek DA, Stock PG, Baekkeskov S. Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. Nat Med 1997; 3(7): 738-43.

[74] Kabelitz D. Apoptosis, graft rejection, and transplantation tolerance. Transplantation 1998; 65(7): 869-75.

[75] Allison J, Georgiou HM, Strasser A, Vaux DL. Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet al.lografts. Proc Natl Acad Sci U S A 1997; 94(8):3943-7.

[76] Powell TJ JR, Streilein JW. Neonatal tolerance induction by class II alloantigens activates IL-4-secreting, tolerogen-responsive T-cells. J Immunol 1990; 144(3):854-9.

[77] Schurmans S, Heusser CH, Qin HY, Merino J, Brighouse G, Lambert PH. In vivo effects of anti-IL-4 monoclonal antibody on neonatal induction of tolerance and on an associated autoimmune syndrome. J Immunol 1990; 145(8):2465-73.

[78] Takeuchi T, Lowry RP, Konieczny B. Heart allografts in murine systems. The differential activation of Th-2-like effector cells in peripheral tolerance. Transplantation 1992; 53(6):1281-94.

[79] Bushell A, Pearson TC, Morris PJ, Wood KJ. Donor recepient icrochimerism is not required for tolerance induction following recipient pretreatment with donor-specific transfusion and anti-CD 4 antibody. Transplantation 1995; 59: 1367-1371.

[80] Pullar CE, Morris PJ, Wood KJ. Altered proximal T-cell receptor signalling events in mouse CD4+ T-cells in the presence of anti-CD4 monoclonal antibodies: evidence for reduced phosphorylation of Zap-70 and LAT. Scand J Immunol 2003; 57(4): 333-41.

[81] Sablinski T, Sayegh MH, Hancock WW, Kut JP, Kwok CA, Milford EL, Tilney NL, Kupiec-Weglinski JW. Differential role of CD4+ cells in the sensitization and effector phases of accelerated graft rejection. Transplantation 1991; 51: 226 - 231.

[82] Claas FH, Roelen DL, van Rood JJ, Brand A. Modulation of the alloimmune response by blood transfusions. Transfus Clin Biol 2001; 8: 315 –317.

[83] Opelz G, Vanrenterghem Y, Kirste G, Gray DW, Horsburgh T, Lachance JG, Largiader F, Lange H, Vujaklija-Stipanovic K, Alvarez-Grande J, Schott W, Hoyer J, Schnuelle P, Descoeudres C, Ruder H, Wujciak T, Schwarz V. Prospective evaluation of pretransplant blood transfusions in cadaver kidney recipients. Transplantation 1997; 63: 964 –947.

[84] Josien R, Douillard P, Guillot C, Muschen M, Anegon I, Chetritt J, Menoret S, Vignes C, Soulillou JP, Cuturi MC. A critical role for transforming growth factor-β in donor transfusion induced allograft tolerance. J Clin Invest 1998; 11: 1920 -1926.

[85] Koga S, Luke PP, Specht SM, Rominski B, Jaquins-Gerstl A, Hoffman RA, Thomson AW, Jordan ML. Inhibition of the allograft response by donor specific blood transfusion: association with reduced local Th-1 cytokines and nitric oxide but enhanced prostaglandinE2 production. Transplantation 2000; 12: 1788–1796.

[86] Brand A. Immunological aspects of blood transfusions. Transplant Immunology 2002; 10: 183 – 190.

[87] Yang C-P, Shittu E, Bell EB. Specific B-cell tolerance is induced by cyclosporin A plus donor-specific blood transfusion pretreatment: prolonged survival of MHC class I disparate cardiac allografts. J Immunol 2000; 164: 2427 - 2432.

Chapter 5

MOTIVATION FOR THE STUDY

Motivation.

The prevalence of diabetes mellitus is increasing rapidly worldwide and in South Africa afflicts many underprivileged population groups. Diabetes, apart from cardiovascular disorders, trauma and cancer remains one of the leading causes of premature death in man.

Stats SA numbers for the top 20 leading causes of death are derived from what is written on death certificates. Most diabetics die of the complications of diabetes so their deaths are attributed to those diseases and not diabetes per se (these amount to about 11-15%). If you add these to deaths due to diabetes (and remove them from the other lists) diabetes comes to the top of deaths due to non-infectious diseases. In the Stats SA top 20 causes of death, the order becomes: 1 and 2 due to trauma, 3 – 5 due to AIDS, TB and pneumonia and in 6th place are diabetes and one of the cardio diseases.

End-stage renal failure and cardiovascular related disorders (i.e. arteriosclerosis, accelerated coronary artery disease) frequently complicate the course of diabetes, despite conventional medical treatment; this has been documented globally over decades. Target-organ failure affects both caucasian and black populations and is not in dispute. This places huge demands on renal dialysis facilities and cardiac units. Many unfortunate individuals afflicted by diabetes with advanced complications, especially in Africa, are sent home to die from a terminal disorder and secondary complications. Many are in the age group of 40 – 60 years. Surgical amelioration of diabetes by pancreatic replacement in selected patients has theoretical and clinical advantages. Hyperglycaemia and need for insulin therapy can be abrogated whilst the graft functions; the daily fluctuations of blood sugar levels are taken care of by the graft. "Diabetics" are then able

to resume a "normal diet" and do not have to fear the danger of coma, hypo- and hyperglycaemia and death. This is beneficial to diabetics. On the other hand, the application of pancreatic transplantation, outside the USA [7697 of 10289 pancreas transplants reported to the International Pancreas Transplant Registry (1998) were done in the USA], has been viewed critically, due to poor clinical results[1,2]. However many persons with complicated diabetes have benefited substantially from pancreas whole organ transplantation[2]. Provided organ rejection can be controlled, the sustainment of pancreatic replacement therapy (as a source of insulin) is undisputed. Indeed many human diabetics have been rendered insulin independent and free from uraemia for periods longer than 10 years after transplantation[3]. A cure for diabetes is yet to be documented but results are encouraging. In the light of this, all new information regarding the potential amelioration of the diabetic state, by β-cell replacement, is a valuable adjunct in the treatment of diabetes mellitus.

Restoration of endocrine function, and the correction of metabolic derangements remains the ultimate goal in the management and treatment of type 1, insulin dependent diabetes mellitus. Pancreatic transplantation has met with increased success (because of improved immunosuppression) and the need for such replacement, especially in diabetics with end-stage renal disease, has grown rapidly, outstripping the supply of donor organs[4,5,6]. A successful pancreas graft, provided rejection is prevented, restores normoglycaemia and reduces many of the serious metabolic complications common to diabetes[7-20].

Islet transplantation in humans is problematic (because of accelerated islet cell rejection) and has not met with the same success as whole pancreas transplantation[20]. This also applies to laboratory models[18]. Isolation of sufficient numbers of viable islets, and other technical aspects,

have been stumbling blocks hampering clinical application. Foetal pancreas transplantation, as a source of endocrine replacement, offers several attractive benefits including ease and safety of transplantation and the potential of the foetal pancreas endocrine component to grow and differentiate[11]. The preferential early development of the endocrine component in the foetal pancreas, counteracts the need for isolation of the islets from exocrine tissue shown to be necessary for successful non-vascularized islet engraftment[11,13,14]. Reversal of chemically induced diabetes in mice and rats by foetal pancreas transplantation is possible and euglycemia can be maintained for long periods provided that adequate immunosuppression is maintained[11].

One of the major stumbling blocks hindering the clinical application of foetal islet transplantation is the fierce rejection of the allogeneic grafts due to the high antigenicity of allogeneic foetal pancreata[21,22]. Mandel (1984) showed that organ cultured foetal pancreas was less immunogenic. Culture resulted in exocrine degeneration resulting in selective survival of islet cells. Islet endocrine cells are less antigenic, possibly because they lack MHC II antigens[23].

New immunosuppressive drugs and treatment protocols have alleviated the diabetogenic side-effects seen with conventional macrolide and steriod based immunosuppression[24,5]. Pancreatic transplantation, for treating type 1 diabetics with complications, has benefited from new immunosuppression protocols[24].

The use, in this study, of established conventional immunosuppressants such as cyclosporine and newer immunosuppressive drugs, like tacrolimus (FK506) and mycophenolate mofetil (MMF), may further contribute to the application of these drugs as immunosuppressants in the preclinical and clinical setting. The use of monoclonal antibodies raised against specific target activation molecules like the CD4 molecules, as biological immunosuppressants, offers attractive potential

alternatives to other immunosuppressants[25]. In this study, the use of monoclonal antibodies against epitopes of cell surface receptor molecules which are associated with alloantigen recognition, will hopefully improve our understanding of the graft rejection process and immunosuppression.

The possibilities of treating diabetes mellitus by implantation of foetal islets, in conjunction with effective immunosuppression, is very exciting and the central theme of this study.

Hypotheses tested and outcomes studied.

In order to test the null hypothesis, the following hypotheses were investigated:

1. Syngeneic foetal RPT is not capable of reversing major clinical, endocrine and metabolic derangements in chemically-induced diabetes in laboratory rats.

2. Engraftment of whole syngeneic and allogeneic foetal pancreas beneath the rodent renal capsule is not viable or durable.

3. Proliferation of engrafted syngeneic foetal pancreas does not occur.

4. Short – medium term engraftment (first 14 days after engraftment) and survival of islet allografts does not occur after allogeneic FRPT in non-diabetic allogeneic rats.

5. Rejection or prolongation of graft survival of FRPT, compared to untreated allografts, does not occur following the administration of the available clinical immunosuppressive agents, W3/25, CsA, FK506 and MMF either alone or in combination.

Expected Research outcomes:

1. That syngeneic whole foetal pancreatic transplantation under the kidney capsule results in the proliferation and maturation of viable endocrine tissue in a laboratory rat model (WAG ⇒ WAG and DA ⇒ DA).

2. Pancreatic allograft (WAG ⇒ SD and DA ⇒ SD) survival (short and intermediate term) in an established laboratory rat transplantation model using conventional immunosuppressants i.e. cyclosporine (CsA), tacrolimus (FK506), mycophenolate mofetil (MMF) and an experimental monoclonal antibody W3/25 (a specific immunosuppressant against the CD4 molecule).

3. The immunosuppressive agents W3/25, CsA, FK506 and MMF (used alone or in combination) prevent allograft rejection and provide for short term endocrine development following FRPT.

Aims and Objectives of study.

One of the goals, [focus and intentions] of this laboratory study in rodents, is to contribute new information to the scientific literature. The potential to "reverse" the diabetic state, was the main stimulus for this study.

The aim of this research project was to study the effect of isogeneic and allogeneic foetal rat pancreas transplantation, as a form of surgical endocrine replacement therapy, in experimental laboratory diabetic and non-diabetic rat models.

The chief objectives of the study were to:

- establish a reliable, chemically induced laboratory, diabetic rodent model,
- assess the efficacy of immunosuppressive agents, including CsA, FK506, MMF and an experimental mouse anti-rat CD4 antibody, to suppress rejection of allogeneic foetal rat pancreatic allografts,
- assess the pharmacokinetic effects of the various immunosuppressants on the immune system by flow cytometric analysis of peripheral blood lymphocytes,
- study and compare the survival and endocrine development following isogeneic and allogeneic foetal rat pancreatic transplantation in diabetic and non-diabetic recipients,
- research, in detail, the histological process after foetal pancreatic transplantation and focus on the degree of rejection following immunosuppression in the allograft model,
- perform morphometric analysis of graft survival and endocrine development following foetal rat pancreas engraftment into both isogeneic and allogeneic laboratory models,
- contribute to the fundamental experimental and theoretical knowledge (endocrine, histological and immunological) of diabetes mellitus and transplantation of foetal islet allografts.

Ethical issues.

A rigid ethical code of conduct has been adhered to according to the "Ethical Considerations in Medical Research Revised Edition MRC" – pages 34 – 39 pertaining to animal research since

registration of Protocol 87/072. In particular the proper use of analgesics, and the administration of anaesthetics according to recognized veterinary practice, has enjoyed high priority. As far as anaesthesia in rats is concerned, this laboratory has found the use of ether, ketamine hydrochloride (Ketalar®), local anaesthesia and chloral hydrate (either singly or in combination) completely adequate for procedures needed in this project. Postoperative warmth is provided by short-term overhead lighting, which also reduces immediate postoperative pain. A register of experimentation reflecting animal number, procedure and outcome is kept and available for inspection.

BIBLIOGRAPHY

[1] Sutherland DER. Report from the international pancreas transplant registry. Diabetologia 1991; 34: 28-39.

[2] Sutherland DER, Cecka M, Gruessner AC. Report from the international pancreas transplant Registry-1998. Transplant Proc 1999; 31: 597-601.

[3] Sutherland DER. Pancreatic transplantation: state of the art. Transplant Proc 1992; 24: 762-766.

[4] Cicalese L, Giacomoni A, Rastellini C, Benedetti E. Pancreatic transplantation: a review. Int Surg 1999; 84(4): 305-12.

[5] Gruessner AC, Sutherland DER, Gruessner RWG. Report of the international pancreas transplant registry. Transplant Proc 1998; 30: 242-243.

[6] Bouwens L. Transdifferentiation versus stem cell hypothesis for the regeneration of islet beta-cells in the pancreas. Microsc Res Tech 1998; 43(4): 332-6.

[7] Yderstraede K.B, Starklint H, Kemp E, Salomon S, Beck- Nielsen H. Transplantation of fetal and neonatal islet tissue: functional and morphological evaluation of free and encapsulated islet grafts. Transplant Proc 1995; 27: 3182-3183.

[8] Yderstraede KB, Henriksen FL, Nielsen T, Rohr N, Larsen KE, Pozza G, Di Carlo V, Socci C, Secchi A, Birkeland SA, Beck-Nielsen H. Insulin independence after allogeneic intraportal islet transplantation: relation to functional tests. Transplant Proc 1998; 30: 317-318.

[9] Weitgasser R, Davalli AM, Napoli R, Capotorto JV, Finegood DT, Bonner-Weir S, Weir GC. Long-term normalization of GLUT 4 protein content in skeletal muscle of streptozotocin-diabetic Lewis rats after islet transplantation. Transplant Proc 1995; 27(6): 3184-5.

[10] Tajra LC, Molina G, Albalate P, Lefracois N, Brunet M, Martin X, Dubernard JM. Early postoperative period: study of metabolic profiles after segmental or total pancreas transplantation. Transplant Proc 1994; 26: 478-479.

[11] Brown J, Danilovs JA, Clarck WR, Mullen YS. Fetal pancreas as a donor organ. World J Surg 1984; 8: 152-157.

[12] Mullen YS, Clark WR, Molnar IG, Brown J. Complete reversal of experimental diabetes mellitus in rats by a single fetal pancreas. Science 1977; 195: 68.

[13] Mc Evoy RC, Hegre OD. Syngeneic Transplantation of Fetal Rat Pancreas II. Effect of Insulin Treatment on the Growth and Differentiation of Pancreatic Implants Fifteen Days After Transplantation. Diabetes 1978; 27 (10): 988 - 995.

[14] Mc Evoy RC, Hegre OD. Syngeneic Transplantation of Fetal Rat Pancreas III. Effect of Insulin Treatment on the Growth and Differentiation of Pancreatic Implants After Reversal of Diabetes. Diabetes 1979; 28: 141 - 146.

[15] Feldman SD, Scharp DW, Lacy PE, Ballinger WF. Fetal pancreas isografts, cultured and uncultured, to reverse streptozotocin- induced diabetes mellitus. J Surg Res 1980; 29: 309-318.

[16] Mandel TE. Organ culture of foetal mouse and foetal human pancreatic islets for allografting. Diabetes 1982; 31 (4); 39.

[17] Du Toit D, Muller C, Page B, Louw J. Foetal Rat Pancreatic Transplantation: Posttransplantation Development of Foetal Pancreatic Iso- and Allografts and Suppression of Rejection With Mycophenolate Mofetil (MMF) and Cyclosporine Based immunesuppression. Microsc Res Tech 1998; 43 (8): 347 –355.

[18] Kemp C.B, Knight M.J, Scharp D.W, Balinger W.F, Lacy P.E. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia. 1973; 9: 486-491.

[19] Kemp JA, Mullen Y, Weissman H, Heininger D, Brown J, Clark WR. Reversal of diabetes in rats using fetal pancreata stored at- 196 C. Transplantation 1978; 26: 260-264.

[20] Hiller WF, Klempnauer J, Luck R, Steiniger B. Progressive deterioration of endocrine function after intraportal but not kidney subcapsular rat islet transplantation. Diabetes 1991; 40(1): 134-40.

[21] Lafferty KJ, Hao L. Fetal pancreas transplantation for treatment of IDDM patients. Diabetes Care 1993; 16: 383 – 386.

[22] Mullen Y. Permanent reversal of experimental diabetes in rats by allogeneic fetal pancreata across minor histocompatibility loci: effects and characteristics of immunosuppression induced by alloantigen, PCH, and ALS treatment. Transplant Proc 1981; 13: 823-825.

[23] Mandel TE. Transplantation of organ- cultured fetal pancreas: experimental studies and potential clinical application in diabetes mellitus. World J Surg 1984; 8: 158-168.

[24] Gummert JF, Ikonen T, Morris RE. Newer immunosuppressive drugs: a review. J Am Soc Nephrol 1999;10(6):1366-80.

[25] Sablinski T, Hancock WW, Tilney NL, Kupiec-Weglinski JW. CD4 monoclonal antibodies in organ transplantation--a review of progress. Transplantation. 1991; 52(4): 579-89.

Chapter 6

LABORATORY MODEL

METHODOLOGY

Diabetic laboratory model (After Brown et al. 1976, Mandel 1984 and Kemp et al. 1978)

Streptozotocin is a diabetogenic agent used to induce a diabetic state in various laboratory animal models. Streptozotocin-induced diabetes has been widely used in pancreas transplantation studies, especially in the rat[1-5].

Induction: Intravenous injection of 50 mg/kg streptozotocin (Sigma Chemical Co, St Louis, USA) in 0.1 M citrate buffer pH 4.0 given into the tail vein of adult rats resulted in diabetes within 2 – 3 days of the injection.

Diabetic state: Streptozotocin induced diabetes shows all the clinical signs associated with an insulin dependent type diabetes with hyperglycaemia, glycosuria, ketonuria, polyuria, and polydipsia. Monitoring of whole blood glucose (Glucometer, Ames, Isando, South Africa) was performed daily and the diabetic animals were supported with insulin (Actraphane®, Novo, Johannesburg, South Africa) as prescribed in table 1.

Pre-transplantation: Pre-transplantation blood glucose levels were tightly controlled with soluble insulin (table 1) to prevent uncontrollable ketoacidosis. Only diabetic animals, with whole blood glucose (WBG) levels ranging between 5.5 and 7.0 mmol/l, were subjected to operation.

Post-transplantation: Whole blood glucose levels were monitored daily and the animals were supported with insulin as per schedule (table 1). Insulin support was only withdrawn once the animal was able to maintain a WBG level of below 8.0 mmol/l for 3 consecutive days without insulin administration. Thereafter WBG levels were monitored weekly.

Non-fasting WBG	Insulin (Actraphane®)
< 10 mmol/l	No insulin
10 – 15 mmol/l	1 Unit
15.1 – 20 mmol/l	2 Units
> 20 mmol/l	3 Units

Table 1: Insulin treatment schedule pre-transplantation and post-transplantation

Histocompatibilty strains tested

WAG to WAG (syngeneic)

DA to DA (syngeneic)

DA to Sprague-Dawley (allogeneic; strong responder)

DA to PVG (allogeneic; inbred strain)

Donor (WAG - inbred strain)

WAG RT1^u inbred rats (Origin: 1924; A.L. Bacharach, Glaxo Laboratories, United Kingdom, inbred from Wistar stock, Gill et al. 1995 in immunology of the rat, Festing 1979 in Inbred strains in biomedical research) were used as donors. Inbred WAG rats, descendants from the original stock, imported from Harlan, U.K. April 1994 were available as specific pathogen free (SPF) rats at our animal facility, Tygerberg Faculty of Health Sciences. Histocompatibilty testing of syngeneic isografts were histologically evaluated (Pilot study - Prof DF du Toit,

Department of Anatomy and Histology) and no rejection of renal subcapsular foetal pancreas transplantation was demonstrated.

Dark Agouti (DA-inbred strain)

DA RTI^{av1}. Inbred for more than 50 generations. Origin unknown, possibly related to COP, a black hooded rat bred by Curtis 1921 at the Columbia University for Cancer Research[6]. Harlan UK received their DA strain in 1979 from the Agricultural Research Centre Cambridge and we imported our stock from Harlan UK in 1998. Our DA rats are kept under specific pathogen free conditions in the animal facility, Faculty of Health Sciences, University of Stellenbosch. The DA is widely used in experimental transplant immunology. Histocompatibilty testing of syngeneic isografts were histologically evaluated (Pilot study, Department of Anatomy and Histology) and no rejection of renal subcapsular foetal pancreas transplantation isografts was demonstrated.

Recipient (SD – outbred strain)

Sprague-Dawley rats (strain initiated by R. Dawley, Sprague-Dawley Company, Madison, Wisconsin, 1925). Species began by mating a hooded male (unknown origin) with 6 albino females (Douredoure strain, from Wistar). The original hooded male was then mated with the female offspring for seven consecutive generations. Thereafter the colony was outbred to develop a stable heterogenous stock. The colony was then closed shortly after its development and no new stock was introduced. The Sprague-Dawley colony at our animal facility was bred from stock bought from Witwatersrand University in 1994. These rats originated from Harlan, U.K. and were direct descendents of the original stock bought from the former Sprague-Dawley company in 1980. Sprague-Dawley rats are large robust albino rats known to be very docile and

tolerate surgery well. The haplotype of outbred rats, like the Sprague-Dawley rats, is variable and are therefore not characterized by Harlan (personal communication with Dr A Deeny, Harlan, U.K.).

PVG (inbred strain)

PVG haplotype $RT1^c$. Black hooded rat. Inbred for more than 70 generations. Originate from Kings College of Household Science then to Lister Institute and to Virol before transfer to Glaxo in 1946. Strain was inbred by Glaxo. Harlan UK received their inbred PVG strain from the Agricultural Research Centre, Cambridge in 1979. PVG rats, imported from Harlan in 1989, have since been kept under specific pathogen free conditions in the animal facility, Faculty of Health Sciences, University of Stellenbosch. PVG rats are very docile rats which tolerate surgical procedures well.

Isograft (syngeneic) controls

WAG foetal pancreata of 16 –18 days gestation were transplanted under the kidney capsule of adult (5 months) male non-diabetic WAG rats. Recipient WAG rats were between 4 and 6 months old and weighed between 350 and 470 g at the time of transplantation. Grafts were harvested at 14 and 30 days post-transplantation and evaluated histologically for signs of rejection. These grafts were used as histological controls and to evaluate graft development and growth.

Allogeneic transplantation (Allografts)

Allograft model: DA $\Rightarrow$ SD (outbred strain). This model severely rejects foetal pancreas allograft in the renal subcapsular space[7]. Foetal pancreata (16 – 18 days gestation) from pregnant

inbred DA rats were transplanted into adult non-diabetic male Sprague-Dawley (outbred strain) rats. All rats were obtained from the central research facility and were bred from specific pathogen free stock. Recipient Spraque-Dawley rats were between 4 and 6 months old and weighed between 430 and 520 g. Animals where then randomized into the experimental groups as shown in table 2.

Allograft model: DA ⇒ PVG (inbred strain). Haplotype $RT1^a$ to $RT1^c$. This model has a delayed response to the allograft antigens. Foetal pancreata (16 – 18 days gestation) from pregnant inbred DA rats were transplanted into adult non-diabetic and diabetic PVG (inbred strain) rats. All rats were obtained from the central research facility and were bred from specific pathogen free stock. Recipient PVG rats were between 4 and 6 months old and weighed between 250 and 280 g. Animals where then randomized into the experimental groups as shown in table 2.

Suitability of the allograft experimental model

High responder allograft model (DA ⇒ Sprague-Dawley): The suitability of the DA ⇒ Sprague-Dawley rat model has been established by this group in various publications[7-14]. Our studies have indicated severe rejection (histocompatibilty barrier) in the WAG ⇒ SD combination when utilizing a "preferred site" of foetal pancreas transplantation[8,9,10,12]. We have demonstrated that, without immunosuppression, rejection ultimately destroys grafts within 4 days of transplantation in these strains.

Inbred strain allograft model: DA ⇒ PVG. Haplotype $RT1^a$ to $RT1^c$. This haplotype combination has been shown to have a low response to the donor antigen resulting in delayed

allograft rejection and allowing for better graft survival[15]. In a pilot study, done in the Department of Anatomy, Faculty of Health Sciences, University of Stellenbosch, we demonstrated that renal subcapsular foetal pancreas allografts were rejected by 14 days post-transplantation if no immunosuppression was used. Immunosuppression, using low dose CsA (2 mg/kg/d) and combination CsA (2 mg/kg/d) and MMF (50 mg/kg/d) therapy, achieved good foetal pancreas allograft survival in the renal subcapsular space over a 30 day period. Stumbles and Mason (1995) have done *in vitro* work on this model and found that, when W3/25 was introduced with the DA antigens, IFN-γ was completely inhibited while IL4 secretion was enhanced[16].

Anaesthesia and alleviation of pain.

All procedures were performed under general anaesthetic, consisting of a combination of di-ethyl-ether inhalation and Ketamine hydrochloride (Brevinaze®, Pharmacare, Port Elizabeth, South Africa) (0.1 – 0.2 ml) intramuscular injection. Dose adjustments were made according to weight. This regimen has proven to be effective by this laboratory over many years. Lignocaine by local infiltration was used, in addition to the general anaesthetic, to overcome spasm of the veins during catheterization.

Intra- and postoperative management of animals.

Once anaesthetized the abdomen was shaven with an electrical hair clipper and the skin surface disinfected with Hibitane® (Zeneca, Woodmead, South Africa) in 70% ethyl alcohol. Animals were kept warm under a heating lamp during the whole operative procedure and dehydration was prevented by injecting 2 – 3 ml of warm sterile Ringer's lactate solution into the peritoneal cavity, prior to closure of the operation wound. Once sutured, the wound was cleaned with

saline, disinfected with Hibitane® and ethyl alcohol and dried with a sterile cotton swab. Chloromex® antibiotic ointment was applied topically to the wound to prevent infection. Ethyl ether was withdrawn after dressing the wound and the animal allowed to regain consciousness. Conscious animals were returned to a clean cage and kept warm under a heating lamp. Animals had immediate access to clean water but food was withdrawn for 24 hours post-transplantation to allow for the secondary ileus. Thereafter animals had free access to food [Standard maintenance rat pellets (Epol S.A.) contained protein, 200 g/kg; carbohydrate, 100 g/kg; moisture, 100 g/kg; fat, 25 g/kg; phosphorus, 8 g/kg; fibre, 50 g/kg; calcium/phosphorus ratio 1.1 - 1.5:1.0; vitamin C, 50 g/kg] and water. Parenteral prophylactic antibiotics [0.2ml Benzyl penicillin (Pharmacare, Johannesburg, South Africa) and 0.1 ml Amikin® (Bristol-Myers Squibb, Bedfordview, South Africa)] were given, by intramuscular injection, for a minimum of 3 days post-transplantation. This strategy was followed to prevent wound and deep intra-abdominal infection occurring after transplantation.

Harvesting of Foetal Rat Pancreata.

Time bred, foetal pancreata of 16-19 days gestation were obtained from pregnant WAG RT1^{v} (Origin: Bacharach 1924 inbred from Wistar stock) females. Time breeding was done by placing a male WAG rat with 4 female WAG rats for a period of 3 days. Mating was timed from the first day the male and females were put together. The pregnant females were anaesthetized with di-ethyl-ether (AR grade, 99% assay, anaesthetic ether, B&M Scientific, Cape Town, South Africa) inhalation and intramuscular injection of 0.1 ml of ketamine hydrochloride 10 mg/ml (Brevinaze®, Pharmacare, Port Elizabeth, South Africa). The abdomen of the donor was disinfected with Hibitane® (Zeneca, Woodmead, South Africa) and ethyl alcohol and the foetuses

were removed by laparotomy and hysterectomy (figures 4 and 5). Each foetus was removed from its amniotic sac and, using the liver as a landmark, the abdomen of the foetus was opened and the pancreas identified using an operating microscope (figures 6 and 7). The foetal pancreas was carefully dissected from the duodenal loop, stomach and spleen, using the operating microscope and fine jewellers forceps (figure 8). Care was taken to prevent inclusion of fat or parts of adjacent organs. Once removed, the foetal pancreas was placed in cold (4°C) RMPI culture medium (Gibco, Inchinnan, Scotland) and kept on ice until required for transplantation (figure 9). Cold ischaemic times were kept as short as possible, usually between 20 and 90 minutes.

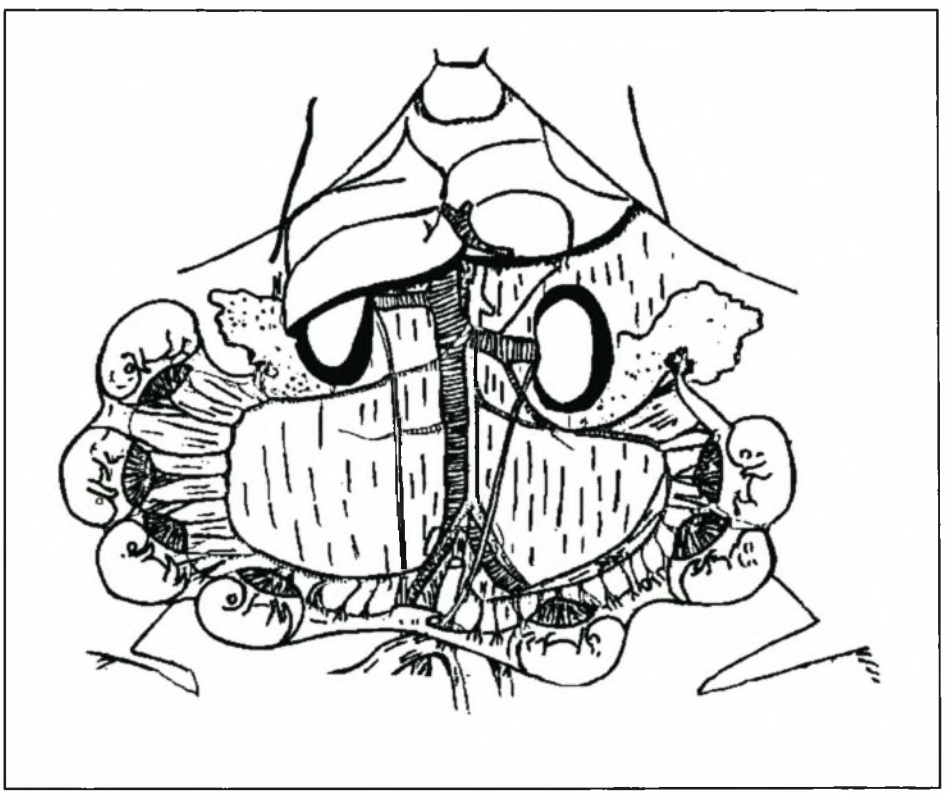

Figure 1: Anatomical drawing showing the position of the pregnant uterus and foetuses in the abdomen of a pregnant rat. The uterus is Y-shaped and may contain as many as 7 –9 foetuses.

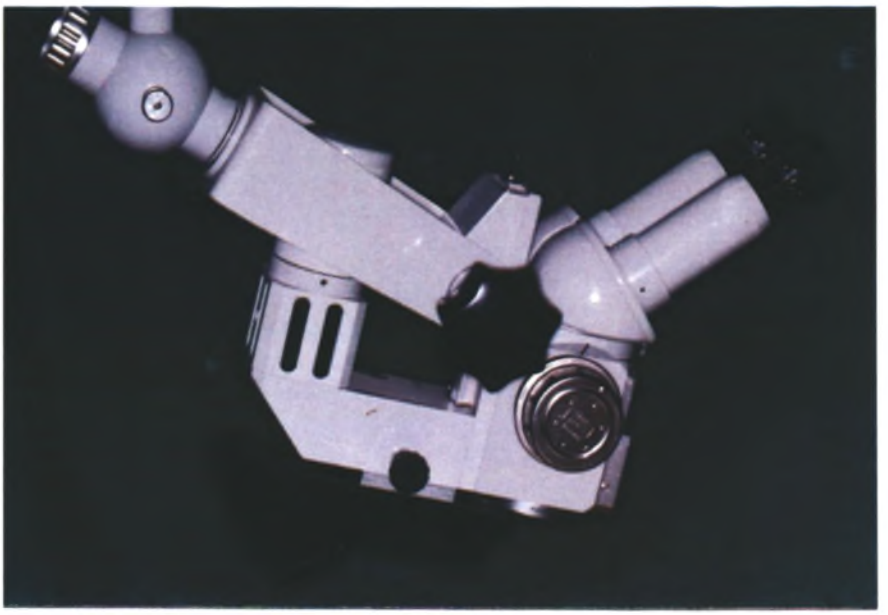

Figure 2: Basic instrumentation required to perform the harvesting of foetal pancreas transplants. An operating microscope Carl Zeiss, Germany, is required for the fine dissection of the pancreas from the attached organs in the rat foetus and for transplanting the harvested pancreas under the kidney capsule.

Figure 3: Standard surgical instruments, including a fine jewellers forceps are used during the surgical procedure.

Figure 4: The uterus of a 17-day gestation pregnant female rat containing several foetuses. The foetuses are surgically removed from the uterus while the female is under general anaesthesia.

Figure 5: A foetus, just removed from the uterus, still in the amniotic sac and with the attached placenta

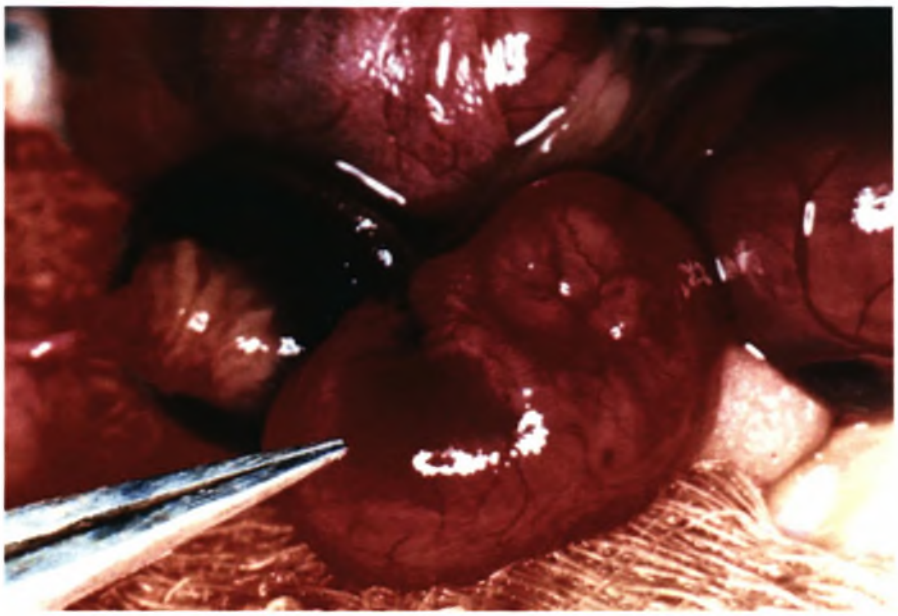

Figure 6: 17-day gestation rat foetus removed from its amniotic sac. The pancreas is dissected from foetus under a surgical microscope.

Figure 7: The liver (arrow) and stomach are apparent anatomical landmarks used to locate the pancreas. The pancreas is carefully dissected from the attached organs under the surgical microscope using fine jewellers forceps.

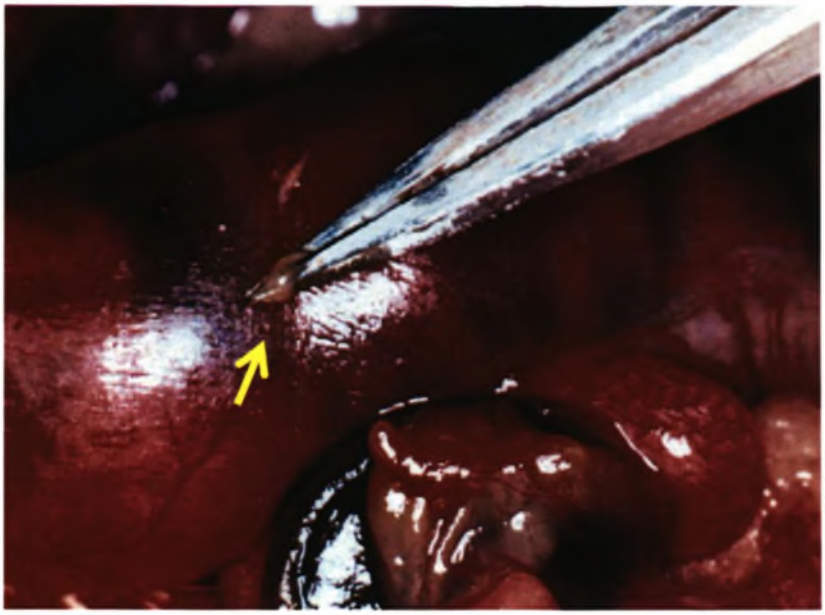

Figure 8: A foetal pancreas (arrow) removed from the foetus is shown in the jaws of a fine jewellers forceps.

Figure 9: After removal the pancreas is kept on ice in RPMI culture medium, until transplantation is affected.

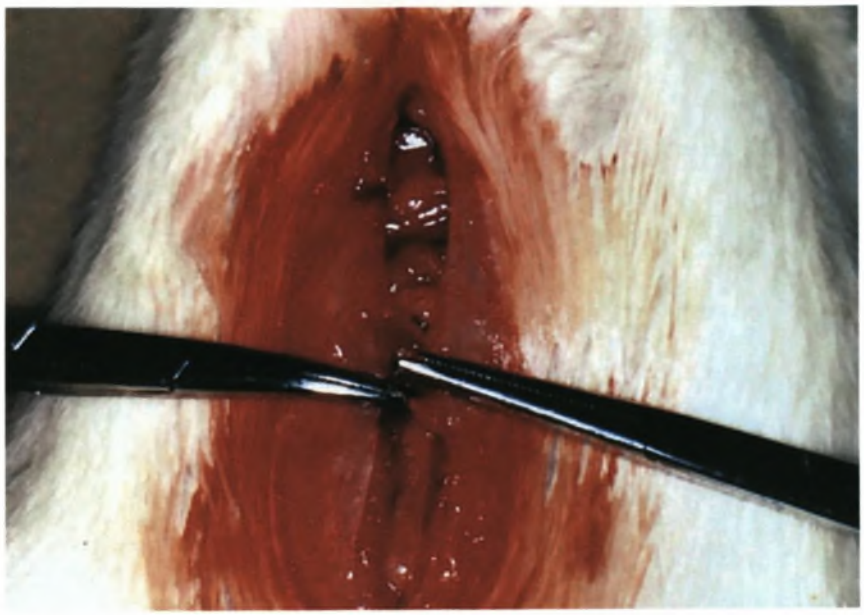

Figure 10: Access to the recipients peritoneal cavity is by midline laparotomy.

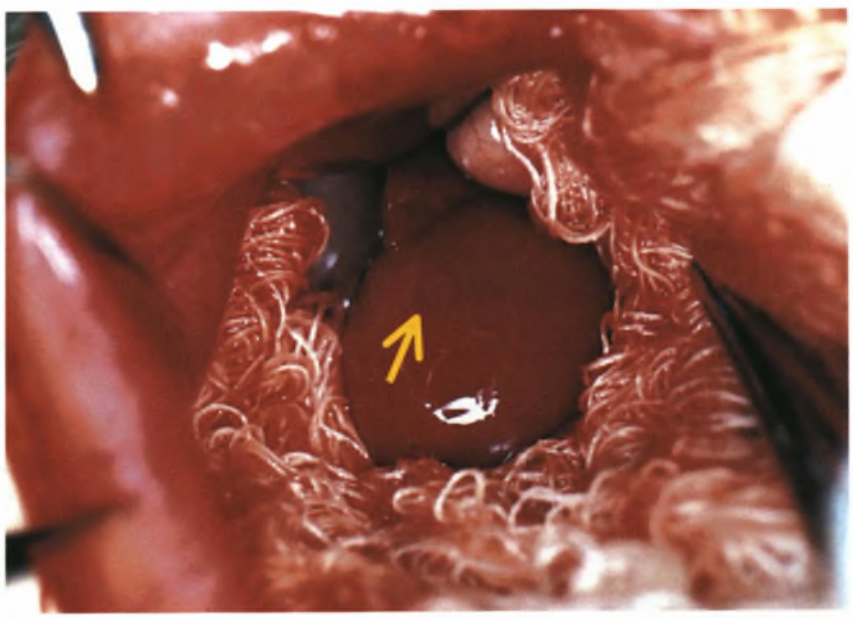

Figure 11: Once exposed, the kidney is gently packed off from the surrounding intestine with gauze. Note the incision (arrow) on the kidney surface through which a subcapsular pocket is prepared for the foetal pancreas transplants.

Figure 12: Anaesthetised adult transplant recipient after closure of the wound. In all cases topical antibiotic creams were applied and parenteral antibiotics (amikin and penicillin) given. Ketalar® is suitable for short term anaesthesia.

Figure 13: Once recovered from anaesthesia, graft recipients were housed in standard laboratory cages (40 x 280cm) containing sterilised woodshavings as bedding

Technique of Transplantation.

A midline laporotomy incision was performed on adult Sprague-Dawley recipient rats under general anaesthesia and the right kidney gently exposed by manually transposing the kidney anteriorly (vertically) in the wound (fig 11). From a technical point of view, the right kidney is easier to utilize for engraftment than the left. In most cases this could be done without disturbing the small bowel or colon. Gauze, moistened with saline, is carefully packed around the kidney. This stabilizes the kidney and protects the surrounding organs (gut). Once the kidney is exposed, an incision of 5-7 mm, just deep enough to incise the capsule, is made on the ventral surface of the kidney. By carefully undermining the cut edge of the capsule with a jeweler's forceps, a subcapsular pocket is created by gently teasing the capsule from the underlying kidney parenchyma. Any bleeding is controlled by gentle external pressure with a cotton tipped ear bud. Once bleeding is controlled, the subcapsular pocket is irrigated with sterile saline and RPMI medium (Gibco, Inchinnan, Scotland). Using a small curved forceps, the harvested pancreas grafts are gently placed, individually, into the prepared pocket. The transplanted pancreata (2 - 4 per recipient) are tightly packed together into the lateral aspect of the pocket and excess fluid evacuated by gentle external pressure, using a cotton bud. To prevent fluid collections, the overlying capsule was fenestrated in the vicinity of the grafts. This allowed evacuation of excess serous fluid that may cause seroma formation. Following engraftment, the subcapsular incision is left unsutured and the kidney is returned to the anatomical position. The abdominal incision is closed by approximating the abdominal muscles and overlying skin with Dexon® 4/0 (Davis & Geck, NJ, USA). The operation wound is then cleaned and disinfected with topical Neosporin® antibiotic ointment (GlaxoWellcome, Midrand, South Africa) application. The ear of the transplant recipient is labelled according to a standardized identity system and parenteral

antibiotic [0.2 ml Benzyl penicillin (Pharmacare, Port Elizabeth, South Africa) and 0.1 ml amikin® (Bristol-Myers Squibb, Bedfordview, South Africa)] is given, by intramuscular injection, to prevent intra-abdominal sepsis after transplantation. The recipient is allowed to recover from the anaesthesia and then returned to a warm cage.

Motivation of the experimental groups used (see table 2).

Syngeneic (DA to DA) foetal pancreas transplantation was used, as an isogeneic control, to study foetal pancreas development and growth in an anatomically displaced environment, under the kidney capsule.

Untreated allograft controls were included to assess the rejection potential of the allogeneic (DA to SD) model in terms of changes in peripheral blood lymphocyte subtypes and histological changes, within the graft, induced by the unmodified rejection process.

Cyclosporine is regarded as the gold standard in terms of short-term immunosuppression, and remains the basis of most immunosuppressive regimens[18-22]. Long-term usage is questionable, due to the inability to prevent chronic rejection in organ allografts[22].

FK506 is structurally different to cyclosporine and binds to a different binding-protein but, like cyclosporine, both block the enzyme calcineurin, an essential enzyme in the activation pathway[23,24,25]. Despite their similarities, FK506 appears to be more potent[26,27,28]. Patients on cyclosporine therapy, with persistent rejection episodes, are often converted to FK506 as rescue therapy[27,29,30]. FK506, also known to interfere with insulin coding, is more diabetogenic than CsA [30] although this is disputed by some studies[31]. Comparing CsA and FK506 monotherapy

groups, in terms of their effect on pancreatic endocrine tissue development, has clinical relevance that can be studied in this developing foetal pancreas model.

Combining CsA or FK506 monotherapy with other immunosuppressants, especially purine inhibitors like aziothiopine or mycophenolate mofetil (MMF), allows for a reduction in toxic dose shedules[7, 9,32,33]. In this study, MMF, used in combination with CsA, was chosen as recent clinical trails have shown that MMF is more effective than azathiopine, if combined with CsA[17].

Reduction of CD4 cells with depleting anti-CD4 monoclonal antibodies such as OX35 and OX 38 in the rat, and L3T4 in the mouse, compromises the immune response in a non-specific way, leading to long-term elimination of $CD4^+$ cells[34]. Provided that sufficient CD4+ cells are eliminated, these animals can theoretically accept allografts indefintely[34, 35].

The use of nondepleting anti-CD4 mAbs, like RIB5/2, have shown that depletion of CD4 cells is not necessary and that these non-depleting anti-CD4 mAbs, induce unresponsiveness and acceptance of the graft, even after therapy has been withdrawn[37-41]. W3/25, a nondepleting anti-rat CD4 mAb raised in mice, has been shown to prevent the induction of experimentally induced autoimmune diseases but it's effectivity in preventing graft rejection in an allogeneic rat model has not been established [10,16,41,42,43,44,45]. Work done *in vitro* by the Mason group in Oxford has shown that W3/25 therapy, at the time of antigen introduction, promotes a Th-2 like response thereby suppressing Th-1 associated cytokines like IFN-γ[16,46]. Although controversial, Th-1 cytokines, and especially IFN-γ, are thought to promote allograft rejection[36,40].

Experimental groups.

The following experimental groups were studied:

GROUPS	TREATMENT	PERIOD (DAYS)	N
	TISSUE CONTROLS		
	Normal adult pancreata to access normal histology and staining techniques (LM, ICC, EM).		10
	17-day gestation transplanted foetal pancreata at 3, 5, 10, 20, 30 days post-transplantation.		25
	SHAM OPERATED CONTROLS		
1	Adult SD rats	14	5
	SYNGENEIC TRANSPLANT CONTROLS (DA⇒ DA)		
2	Controls – no immunosuppression	14	8
	ALLOGRAFTS (DA ⇒ SD) (Histocompatibilty barrier – Outbred strain)		
3	Untreated controls (unmodified rejection)	14	10
4	CsA 5 mg/kg/d - i.m.i. daily for 14 days	14	10
5	MMF alone (50 mg/kg/d) – oral gavage daily for 14 days	14	7
6	CSA (5 mg/kg/d) and MMF 50 mg/kg/d – i.m.i. and oral gavage daily for 14 days	14	5
7	CSA (5 mg/kg/d) and MMF 50 mg/kg/d – i.m.i. and oral gavage daily for 30 days	30	7

8	W3/25 200 µg/d (purified from ascites) - i.p.i. daily for 14 days	14	8
9	W3/25 500µg/d (purified from ascites) - i.p.i. daily for 14 days	14	6
10	W3/25 500 µg/d (purified from ascites) - i.p.i. daily for 30 days	14	6
11	DST 1x 3 ml i.v.i. for 14 days	14	5
12	DST 1x 3 ml i.v.i. and CsA 5 mg/kg/d for 5 days, withdrawn for 9 days	14	5
13	DST 1x 3 ml i.v.i. and W3/25 500 µg/d i.p.i. for 5 days, withdrawn for 9 days	14	6
14	DST 1x 3 ml i.v.i. combined with W3/25 500 µg/d i.p.i. and CsA5mg/kg/d for 5 days, withdrawn for 9 days	14	7
15	DST 1x 3 ml i.v.i. combined with W3/25 500 µg/d i.p.i. and CsA5 mg/kg/d for 5 days, withdrawn for 25 days	30	12
	ALLOGRAFTS (DA ⇒ PVG) (Histocompatibilty barrier – Inbred strain)		
16	Untreated controls	14	5
17	CsA (5 mg/kg/d) i.m.i. daily for 14 days	14	6
18	MMF alone (50 mg/kg/d) – oral gavage daily for 14 days	14	5
19	CSA (5 mg/kg/d) and MMF 50 mg/kg/d – i.m.i. and oral gavage daily for 14 days	14	6
20	CSA (5 mg/kg/d) and MMF 50 mg/kg/d – i.m.i. and oral gavage daily for 30 days	30	6
21	W3/25 200 µg/d (purified from ascites) - i.p.i. daily for 14 days	14	6
22	W3/25 200 µg/d (purified from ascites) - i.p.i. daily for 30 days	30	6

23	DST 1x 3 ml i.v.i. for 14 days	14	5
24	DST 1x 3 ml i.v.i. and CsA 5 mg/kg/d for 5 days, withdrawn for 9 days	14	5
25	DST 1x 3 ml i.v.i. and W3/25 500 µg/d i.p.i. for 5 days, withdrawn for 9 days	14	5
26	DST 1x 3 ml i.v.i. combined with W3/25 500 µg/d i.p.i. and CsA5 mg/kg/d for 5 days, withdrawn for 9 days	14	5
27	DST 1x 3 ml i.v.i. combined with W3/25 500 µg/d i.p.i. and CsA5 mg/kg/d for 5 days, withdrawn for 25 days	30	8
	DIABETIC GROUPS		
	Control groups		
28	WAG normal non-diabetic controls		5
29	SD normal non-diabetic controls		5
30	WAG diabetic controls, STZ 50 mg/kg induced diabetes	30	5
31	SD diabetic controls, STZ 50 mg/kg induced diabetes	30	5
	Isogeneic group		
32	WAG ⇒ WAG, FRPT, STZ 50 mg/kg induced diabetes	30	6
	Allogeneic group		
33	DA ⇒ SD, FRPT, STZ 50 mg/kg induced diabetes, DST 1x 3 ml i.v.i. combined with W3/25 500 µg/d i.p.i. and CsA5 mg/kg/d for 5 days, withdrawn for 25 days	30	10

Table 2: Experimental Transplantation Groups

Immunosuppressants.

Choice and motivation for immunosuppressants

Cyclosporine has revolutionized organ transplantation[48-51]. Multi-organ transplantation has benefited from the introduction of cyclosporine[17,18,51,52,53,54,55]. Cyclosporine binds to the intracellular protein, cyclophillin, and the resulting complex, inhibits calcineurin, an enzyme required for early T-cell activation gene expression, particularly IL-2[22,23,56]. The pharmacokinetics and pharmacodynamics of CsA has been thoroughly researched and has been greatly responsible for our present understanding of the rejection process[22]. Despite the advent of newer immunosuppressive drugs such as tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil, cyclosporine remains the gold standard for immunosuppression[17,18,51,52, 53,54, 55, 57]

Tacrolimus (FK506) is a new immunosuppressive drug that binds to a different intracytoplasmic protein called FK-BP (FK binding protein)[23,25,28]. Like CsA, FK506 also binds to and inhibits calcineurin, a Ca++ dependent serine threonine phosphatase that plays a central role in IL2 promotor encoding following T-cell activation[23,24,25,57]. The binding sites of the CsA and FK506 complexes are not identical but overlap significantly thus causing a similar inhibition of early T-cell activation gene expression[23,25,24,58]. Despite their similarities FK506 is claimed to be 100 times more potent than CsA[59,60].

Mycophenolate mofetil (MMF) inhibits inosine monophosphate dehydrogenase, a rate-limiting enzyme in de novo purine synthesis[23,25]. Activated lymphocytes require this pathway for proliferation while most other cells can use the alternative salvage pathway of purine synthesis for normal cell division[25,49,57,61,62,63,64,65]. MMF acts synergistically with CsA allowing for dose

lowering of CsA in sensitive patients[32,49,53,54,57,66]. Due to its selective purine inhibition of activated lymphocytes, MMF might enhance long-term graft acceptance or even induce tolerance[67 68].

The graft rejection process is initiated by the CD4 T-helper cells which, on recognition of a foreign antigen, produce and secrete cytokines which further activate effector cells in an auto- and paracrine manner[70-76]. Blocking or preventing the CD4 activation signal, with an anti-CD4 monoclonal antibody, alters the immune response to a more tolerant Th-2 like response leading to prolongation of the graft survival[74-83]. The anti-rat CD4 monoclonal antibody W3/25 has been shown to induce a Th-2 like response on alloantigen stimulation in an *in vitro* mixed lymphocyte reaction[16,44,45,84]. W3/25 treatment, as far as we know, does not inhibit/interfere with the activation cascade, as does CsA, FK506 and MMF, but may modify the response to the alloantigen which has a greater likelihood of inducing a state of acceptance or tolerance of the graft[36,70,73]

Introduction of donor derived antigens e.g. donor specific transfusions, under cover of anti-CD4 monoclonal antibody treatment, has been shown to induce donor specific tolerance in various rodent laboratory transplantation models[85-88]. Saitovitch *et al.* showed that if DST is given under cover of anti-CD4, 28 days prior to transplantation, rats accept their grafts without further immunosuppression**Error! Bookmark not defined.**[84]. In this study 3 ml of DA heparinised venous blood was transfused into the recipient via the lateral tail vein.

The use of a short course of CsA, in combination with DST, has also been shown to promote donor specific unresponsiveness in rats by tolerizing B-cells[85].

The combination of DST, W3/25 and CsA, to establish donor specific foetal rat pancreatic graft acceptance, is an unique immunosuppression protocol which combines the potent effect of anti-CD4 and DST with the more controversial tolerogenic effect of short course CsA.

Cyclosporine (CsA)

Sandimmune® 50 mg/ml (Novartis, Kempton Park, South Africa), was administered intramuscularly into the gluteus muscle. To ensure adequate immunosuppression, the parenteral route of administration was selected. The injection site was rotated daily and the dosage calculated according to the daily weigh. Doses used were 2 mg/kg/d, 5 mg/kg/d and 10 mg/kg/d. A dose of 2 mg/kg/d was found to be the minimum effective dose to prevent foetal pancreas allograft rejection by this laboratory[7]. CsA treatment was commenced on the day of transplantation.

Mycophenolate Mofetil (MMF)

MMF, supplied by Roche Pharmaceuticals, was made up in 1% carboxymethyl cellulose (Fluka Chemie, Buchs, Switzerland) to a concentration of 50 mg/ml. The MMF solution was given at a dose of 50 mg/kg/d, by oral gavage. The supplier does not recommend parenteral administration. A dose response curve (pilot study) showed that MMF caused severe haemorrhage of the intestine, especially the lower portion of the small intestine and the large intestine, if the daily dose exceeded 100 mg/kg/d. At a dose of 300 mg/kg/d, 100% of the recipients had died by day five. At 200 mg/kg/d most of the recipients had severe malaena (black stools) by day five and continued to lose weight rapidly. Doses varying between 40 mg/kg/d (rat) and 80 mg/kg/d (mouse) are needed to suppress allografts in experimental models (Hao et al. 1992). Knechtle et al. 1992 showed that 20 mg/kg/d was a sub-optimal dose in rats. In our studies we found that the

dose could be safely increased to 100 mg/kg/d. MMF was used either alone or in combination with CsA (2 mg/kg/d). MMF treatment started on the day of transplantation.

Mouse anti-rat CD4 monoclonal antibody - clone W3/25.

The choice of W3/25 as immunosuppressant:

The W3/25 hibridoma has proved itself as a specific mouse anti-rat CD4 monoclonal antibody [86] [87] [88]. It is now commercially available for immunophenotyping of rat CD4 lymphocytes (Caltag cat. Nr. MR 5100). W3/25, a non-depleting mouse IgG1 isotype antibody, binds to the domain 1 of the rat CD4 molecule[16,44]. OX38, a depleting anti-rat CD4, mouse IgG2b isotype, monoclonal antibody which binds to the same domain as W3/25[89], has also been shown to effectively prolong graft survival[34,37,38,70]. W3/25 hibridoma is produced in the department of Medical Biochemistry, University of Stellenbosch and is readily available.

Preparation of monoclonal antibody (method after Mason et al. 1980; Parham et al. 1982 and Johnstone et al. 1996[90,91,92]):

W3/25 hybridoma cells were kept frozen in Eppendorf tubes under liquid nitrogen in a cryo-protectant containing dimethyl sulfoxide (Sigma, St Louis, USA). These cells were then rapidly defrosted in a 37 °C water bath, the cryoprotectant diluted out in 50 ml RPMI (Gibco, Paisley, Scotland) and foetal calf serum (Flow Laboratories, UK). The cells were then recovered by gentle centrifugation ±800 rpm and resuspended in 10ml culture medium (RPMI and 1% calf serum) in a 10 ml tissue culture flask. Cells were then cultured in a CO_2 incubator overnight and checked for viability and growth. Provided there were enough viable cells they were diluted 1:5 or 1:10 and allowed to grow in the incubator for a further 2-3 days whereafter they were further diluted 1:5 in 50 ml culture medium (RPMI and 1% FCS). Just before the cells reached

confluency they were divided into 2x 50 ml culture flasks and cultured for a further day or two. The cells were then gently centrifuged at 800 rpm at 4 °C, the supernatant removed and stored. The cells were washed in 5 ml sterile PBS, counted in a haemocytometer and the cell suspension diluted to a final concentration of $\pm 3.0 \times 10^6$ /ml and kept on ice.

Preparation of Balb/c mice for hybridoma cell inoculation (method after Johnstone et al. 1996 [92]).

5 – 10 days prior to inoculation of mice with the W3/25 hybridoma, each mouse received 0.5 ml pristane (2,6,10,14-tetramethyl-pentadecane, Sigma, St Louis, USA) by intraperitoneal injection. This gave sufficient time for the recipient mice to develop a reactive ascites/exudate, which supplies essential nutrients to the hybridoma cells.

Inoculation of the pristane primed mice with hybridoma.

5 – 10 days after the mice have been pristane primed, each mouse received an intraperitoneal injection with 0.5 ml or 1.5×10^6 of the cell suspension. If ascites formed, the abdomen became visibly distended within 7 to 10 days. With time the abdomen distended, the hair of the mouse straightened and tachypnoea occured (rapid breathing).

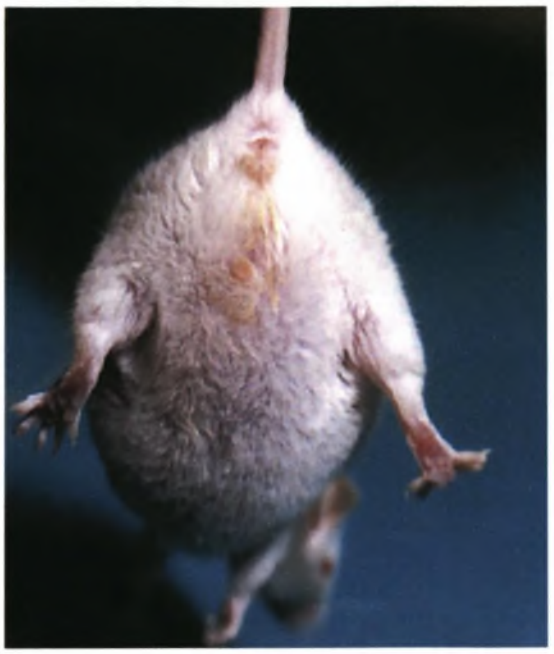

Figure 14: A Balb/c mouse, inoculated with W3/25 hybridoma cells 10 days previously. The production of Ig containing ascites by the inoculated W3/25 cells has induced abdominal distention.

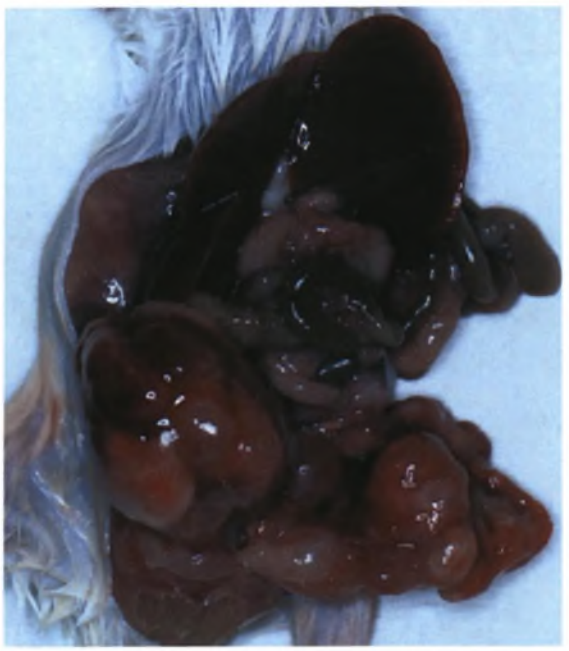

Figure 15: The W3/25 immunoglobulin-secreting tumor, growing in the abdomen. The ascitic fluid was collected by laparotomy.

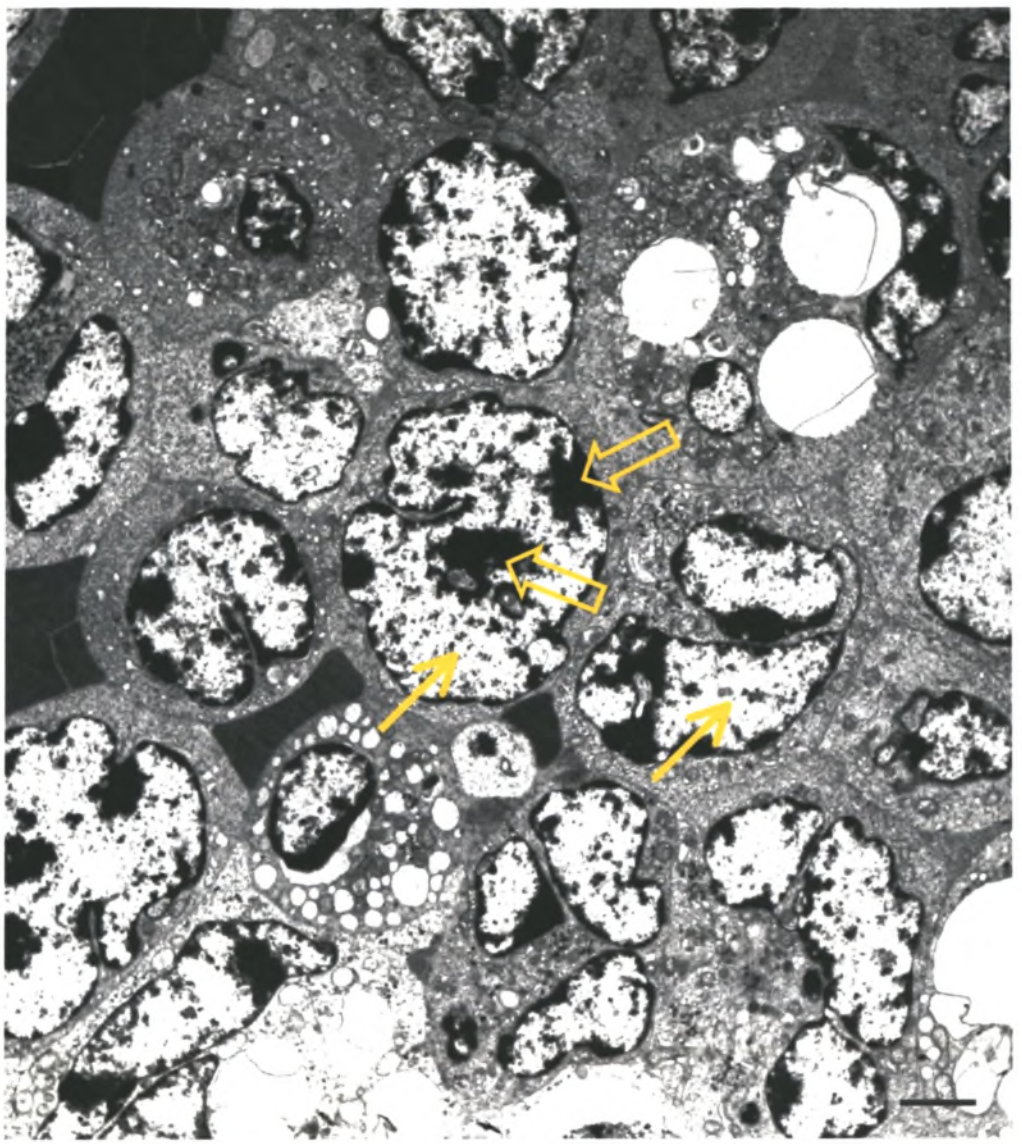

Figure 16: An electron micrograph (x3500) of the W3/25 immunoglobulin-secreting tumour cells show characteristics of active protein secretion including the presence of euchromatin nuclei (→), multiple nucleoli (⇨) and rough endoplasmic reticulum. Scale bar = 2.9 μm.

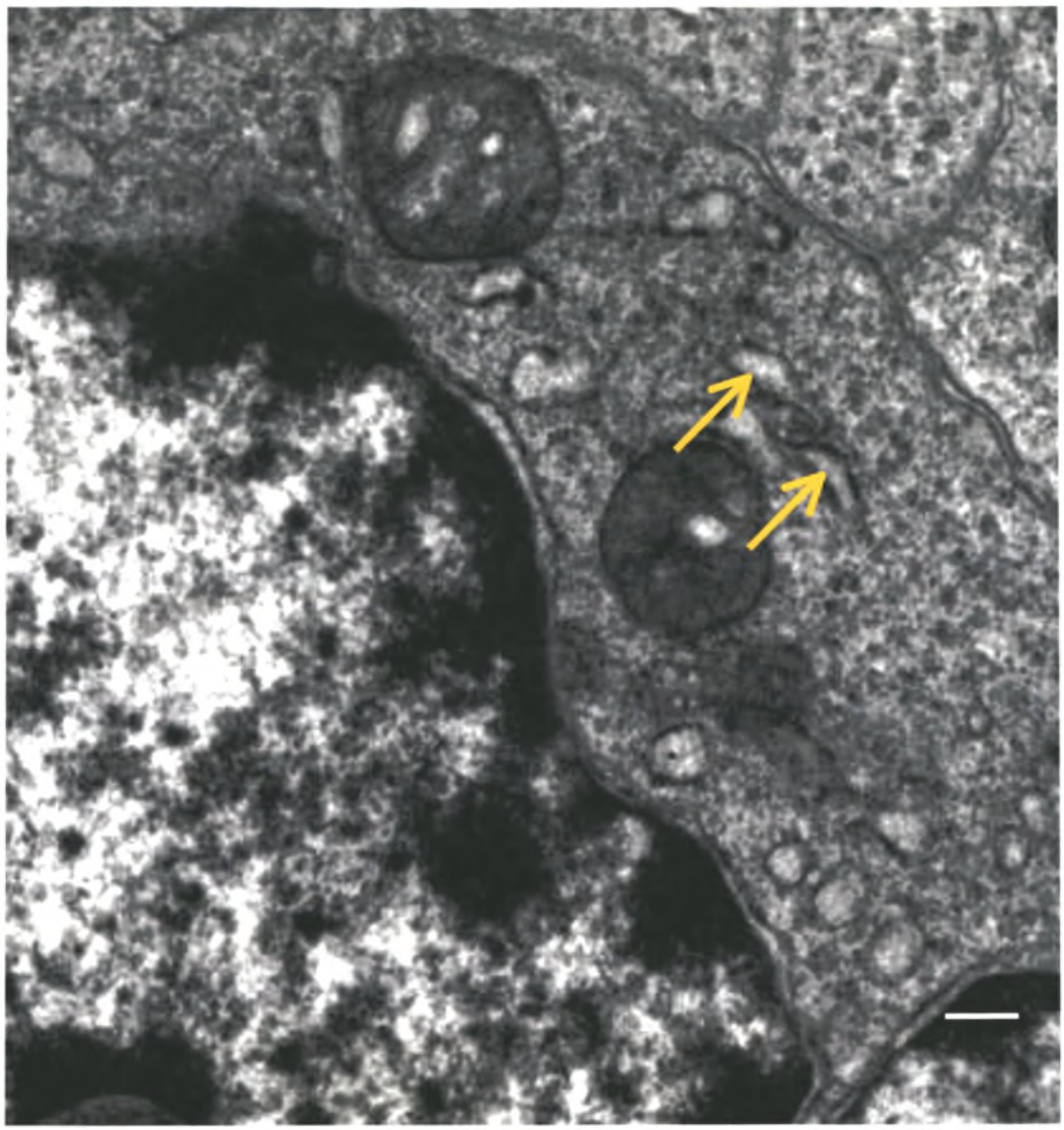

Figure 17: A higher magnification electron micrograph (x10000) shows a W3/25 tumor cell containing rough endoplasmic reticulum with dilated cisternae (→). Scale bar = 1.0 μm.

Once the mice had developed optimal ascites they were euthanased with CO_2 and sterilized by immersion in 70% alcohol. The abdominal skin was carefully removed and the abdominal cavity

entered by a small incision. Ascites, containing the W3/25 monoclonal antibody, was then collected in sterile 50 ml Falcon tubes (Becton Dickinson, Cedex, France) and kept on ice.

The ascites was gently centrifuged at 800 rpm, the supernatant decanted into another sterile tube and 1 mMol sodium azide (BDH Chemicals, Poole, UK) added to the supernatant as a preservative. The cells from the ascites were resuspended with sterile PBS, the concentration readjusted to $1 - 1.5x10^6$ cells, and injected into the next generation of pristane primed mice.

In our experience with ascitic mice there is a low yield from the *in vitro* cell cultured W3/25 hybridoma, but the yield improved dramatically once the cells were transferred from mouse to mouse. Other workers in the field have also seen this with a variety of hybridomas (Mrs H Veenstra of the Department of Medical Biochemistry, Faculty of Health Sciences, University of Stellenbosch, Tygerberg: personal communication).

Precipitation of W3/25 IgG from ascites (After Parham et al. 1982 [91]).

Once sufficient ascites was collected, the fluid was pooled and centrifuged at 10000xG for 30 minutes in order to remove the smaller particulate matter. The volume was accurately measured, 18% w/v Na_2SO_4 (BDH Chemicals, Poole, UK) was added during constant stirring on a magnetic stirrer. The Na_2SO_4 and the ascitic fluid were allowed to incubate for 30 minutes at 37°C and then centrifuged at x8000 rpm for 15 minutes to collect the precipitate. The precipitate was redissolved, in distilled water, to 33% of the initial volume. 18%w/v Na_2SO_4 was again added under constant stirring and incubated at 37°C for a further 30 minutes. The suspension was centrifuged at x8000 for a further 15 minutes as described previously, but the supernatant was retained. The pellet was dissolved in distilled water to 20% of the initial volume. The

preparation was then dialysed using Spectrapor® membrane tubing (Spectrum Medical Industries Inc, Los Angeles, USA) against TBS pH 7.4 (25 mM Tris HCl and 0.05 M NaCl) at 4 °C. The TBS was changed every day until the conductivity of the buffer was the same as the starting buffer.

An ion exchange column was prepared with DEAE cellulose microbeads (Whatman DE-32, Whatman International Ltd, Maidstone, UK) by stirring the cellulose into x15 w/v 0.5 N HCl, and allowed to stand for 30 minutes, then washed with distilled water until the effluent reached an intermediate pH of 4.0. The cellulose microbeads were recovered and stirred into x15 w/v 0.5 NaOH, and left to react for a further 30 minutes. Thereafter the beads were rinsed with distilled water until the effluent reached an intermediate pH of 8.0. A final rinse was done in a beaker and the supernatant poured off to remove the fine particles. The cellulose was stirred into a beaker with TBS (25 mM Tris HCl pH 7.4 and 0.05 M NaCl). The cellulose was recovered and the column loaded (20 ml DEAE cellulose is sufficient for 50 ml ascites). The column was equilibrated by running TBS (25 mM Tris HCl pH 7.4 and 0.05 M NaCl) through the column overnight or longer until the pH and conductivity of the buffer passing through the column was the same as the starting buffer. The dialysed preparation was allowed to slowly pass through the column while collecting the fluid, in 4 – 5 ml fractions. The concentration of each of the fraction was calculated using a Beckman Spectrophotometer, set at 280 nm absorbance. The fractions containing Ig were pooled and dialysed using Spectrapor® membrane tubing (Spectrum Medical Industries Inc, Los Angeles, USA) against 5 mM Tris-HCl, pH 7.5. The precipitate (IgM) was then removed by centrifugation and the supernatant dialysed, once again, against PBS pH 7.4 at 4 °C overnight. The concentration of the purified Ig was determined spectrophotometrically at

280 nm and then aliquoted and frozen in suitable volumes. The antibody was diluted with sterile PBS to give a final working dilution for injection as shown per the experimental groups.

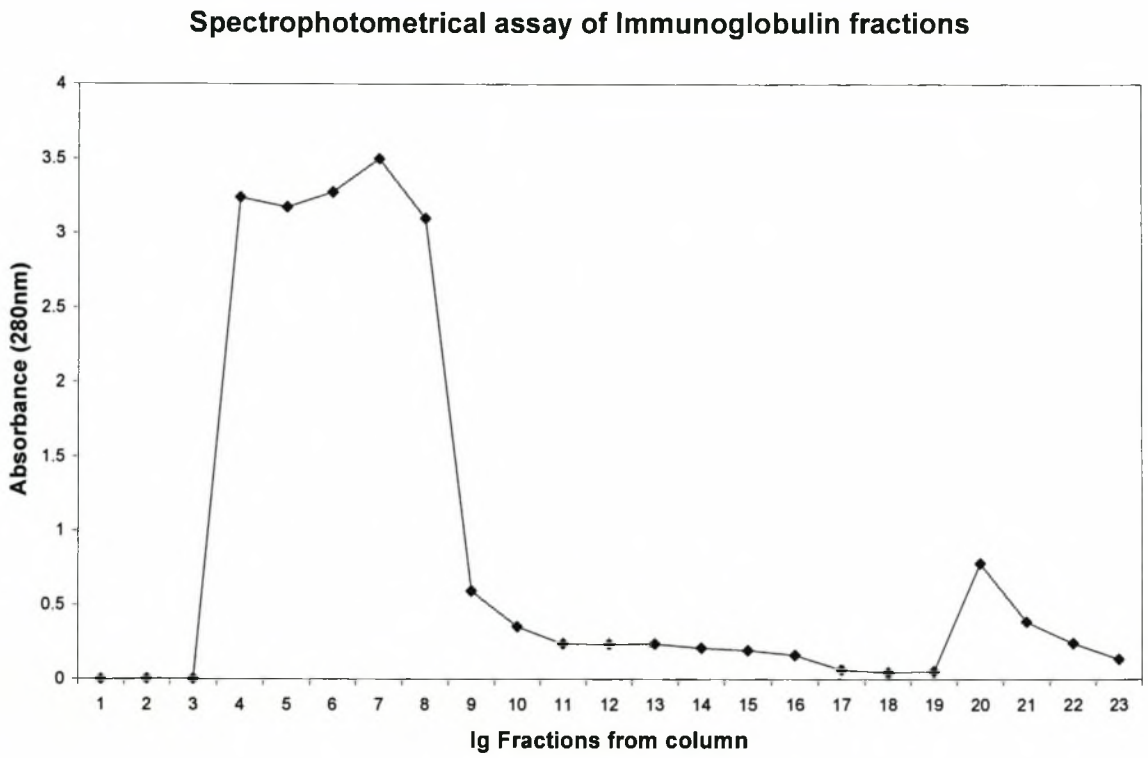

Figure 18: 4 –5 ml fractions assayed spectrophotometrically at 280 nm produced a major peak of IgG between fractions 3 and 9. Increasing the salt concentration of the buffer to 100 mM, in fractions 17 to 22, yielded a further small peak. In this case fractions 3 to 9 were pooled to give a combined optical density of 3.139 and an IgG concentration 2.2 mg/ml (OD/1.43 = mg / ml).

Proteins and enzymes such as albumin and proteases stick to the DEAE cellulose microbeads and are removed from the ascitic fluid. A purified IgG from the major peak was injected into the

rats. This ensures that no unnecessary xenoantigen (mouse proteins) were inoculated along with the W3/25 monoclonal IgG.

Flow Cytometric techniques (Methods prescribed by Becton Dickinson data sheet [93]):

Peripheral Whole Blood.

T-Lymphocytes, both CD4 and CD8 are known to play an central role in allograft rejection [64]. Immunosuppressants target these effector T-cells by preventing transcription of activating cytokines and DNA synthesis[23,25,94]. Monoclonal antibodies target specific activating molecules e.g. CD4 thereby interfering with normal activation following antigen recognition[36,37,38,40,**Error! Bookmark not defined.**,95,96]. Peripheral blood T-lymphocytes mirror the effect of the immunosuppressants used thereby allowing us an easy and accurate method of monitoring the effect of these immunosuppressants on T-lymphocytes[94,97,98,99]. These results can then be correlated with graft survival.

Monitoring of peripheral blood lymphocytes

Peripheral blood was collected via the tail vein in a heparinized Eppendorf tube and kept at room temperature. 100 µl of the whole blood was pipetted into a 3 ml Falcon tube and 30 ul tissue culture supernatant containing the monoclonal antibody (approximately 10 µg Ig/ml) was added, vortex mixed and incubated at 4 °C (on ice) for 30 minutes. The tissue culture supernatants used included OX34 (CD2), W3/25 (CD4), OX8 (CD8) and OX39 (CD25). 3G8, an anti-human CD16 mAb was included as a non-specific control. All supernatants were cultured from the hybridoma bank of the Department of Medical Biochemistry, University of Stellenbosch. Specificity of the supernatants was determined on rat thymocytes by flow cytometry. Following incubation with the primary antibody, unbound antibody was removed by three washes in PBS,

pH7.4, and centrifugation (5minutes at 1500 rpm). Excess PBS was discarded and 30ul of diluted (1:250) secondary FITC conjugated Goat anti-mouse IgG, affinity purified and absorbed against rat serum (Kirkegaard & Perry Laboratories Inc, cat no. 02-18-15, Gaitherburg, USA) antibody added, vortex mixed with the blood and incubated for 30 minutes at 4 °C in the dark at room temperature. Following the secondary incubation the red blood cells were removed by lysing with FACS® lysing solution (Becton Dickinson, San Jose, USA) 2 ml diluted 1:10 in distilled water, for 10 minutes at room temperature. The cells were then washed once more with PBS, centrifuged at x1500 rpm and resuspended in 250 ul PBS pH 7.4 containing 1% formaldehyde (BDH Chemicals Ltd, Poole, UK). The specimens were either read directly or stored overnight at 4 °C before analysis with the FACScan (Becton Dickinson, San Jose, USA).

Determination of bound W3/25 following in vivo injection of W3/25 (Groups 10 – 14).

To determine the amount of surface CD4 molecules that were bound in vivo, by W3/25, 100µl whole blood from recipient rats was washed in PBS and 30 ul diluted secondary FITC conjugated Goat anti-mouse IgG (Kirkegaard & Perry Laboratories Inc, cat no. 02-18-15, Gaitherburg, USA) antibody added, vortex mixed and incubated in the dark. The red blood cells were lysed with FACS® lysing solution (Becton Dickinson, San Jose, USA) and the samples washed with PBS, centrifuged at x1500 rpm and resuspended in 250µl PBS pH 7.4 containing 1% formaldehyde (BDH Chemicals Ltd, Poole, UK). The percentage surface CD4 molecule bound by W3/25 could be determined by correlating the difference in positivity between these tubes and the tubes in which W3/25 was added.

Flow Cytometry

Flow cytometrical techniques that were used were as prescribed and published in standard flow cytometry textbooks and manufacturers training manual[105-108]. Specimens were analysed on a FACScan® using Lysys II® software (Becton Dickinson Immunocytometry systems, San Jose, USA) and a FACSCalibur® (Becton Dickinson Immunocytometry systems, San Jose, USA) using CellQuest™ software version 2.01 (Becton Dickinson Immunocytometry systems, San Jose, USA). A lymphocyte gate was set using forward scatter versus side scatter, in a linear mode, and only cells within the lymphocyte gate analysed. Fluorescent channel 1 (FL1) set in logarithmic mode was used to detect positive cells. CD4 (W3/25) positive cells as a percentage of positive CD2 (OX34) cells, within the lymphocytes gate, was found to be the most reliable method of monitoring the changes within the lymphocyte subsets. OX8, the monoclonal used to identify the CD8 lymphocyte gave less separation between positive and negative peaks and therefore, produced less consistent results.

Immunofluorescence

This was used to establish the phenotypes of the lymphocytes infiltrating the grafts, following various immunosuppressive protocols, in snap frozen sections using the same monoclonal antibodies W3/35 (CD4), OX8 (CD8), OX34 (CD2) and OX39 (CD25) as used in the immunophenotyping of the peripheral blood lymphocytes. These antibodies were selected to establish the lymphocyte phenotype i.e. T-cells of either CD4 or CD8 lineage and state of activation by CD25 expression, present in the grafts.

Immunofluorescence technique: (Methods after Herrington et al. 1992; Bancroft et al. 1990; Pharmingen Technical Protocols[104,105,106]).

Tissue was embedded and orientated in Tissue Tek® OCT™ embedding compound (Sakura Finetek, Torrance, USA), snap frozen with liquid nitrogen onto a piece of cork. Frozen sections of 5-10 μm were cut on an American Optical Cryo-cut® microtome, mounted onto lysinized slides and allowed to air dry for 30 minutes. Air dried sections were briefly fixed in absolute acetone for 5 minutes at room temperature and allowed to air dry for a further 30 minutes. Once dried, sections were washed in PBS pH 7.4, excess PBS blotted from the slides. The sections were then covered by the primary antibody supernatant of W3/35 (CD4), OX8 (CD8), OX34 (CD2) and OX39 (CD25) for 30 minutes, in a damp chamber to prevent drying of the antibody onto the sections. After incubation, primary antibody was drained from the section, the section jet washed with PBS and washed in 3 changes of PBS, by immersion and magnetic stirring for 5 minutes each. The slides were then removed from the PBS; the excess buffer drained and blotted from the slides and fluorescein conjugated secondary rabbit anti-mouse IgG (Kirkegaard & Perry Laboratories Inc, cat no. 02-18-15, Gaitherburg, USA) diluted 1:10 with PBS applied to the sections. The sections were incubated in a moist chamber in the dark for 30 minutes. Following incubation the secondary antibody is the drained from the section, jet washed and washed in PBS as described earlier. Once excess buffer was removed the sections were mounted in PBS buffered glycerin. Sections were viewed with a Fluorescent Microscope (Carl Zeiss, Jena, Germany) fitted with a mercury vapour lamp and blue exciter filter (450 – 490 nm) using x25 and x63 objectives.

Light Microscopy: (Technique After Bancroft et al. 1992[105]).

This was used, histologically, to evaluate and score the harvested grafts in terms of survival and development. Rejection, both in terms of graft destruction and infiltration by inflammatory cells, was evaluated.

All tissues were fixed in 10% formaldehyde in phosphate buffered saline pH 7.4 for 6-12 hours and processed overnight in an automatic tissue processor (Shandon Eliott Duplex processor, Runcorn, England), through ascending concentrations of ethyl alcohol (70%, 2 x 96% and 3x 100%) and xylene into molten paraffin wax. Tissue was then embedded in paraffin wax and 5-7 μm sections cut on a rotary microtome (Jung, Heidelberg, Germany). Sections were floated out on a waterbath (±40 °C), placed onto a glass slide, baked in a 70 °C incubator for 30 minutes prior to staining.

Stained sections were evaluated on a Leitz Laborlux microscope (Ernst Leitz Wetzlar, Wetzlar, Germany) using x4, x10, x20, x 40 and x100 oil objectives.

Haematoxylin and Eosin (H&E) staining: (Technique After Bancroft et al. 1992[105]).

This was used to evaluate and differentiate between the different tissue elements including islets of Langerhans, exocrine acinar tissue and ducts. Changes in graft morphology i.e. fibrosis, fat deposition and infiltration by mononuclear cells can be scored using this stain.

Cut sections were baked for one hour in a 70°C incubator to facilitate effective adherence to the slide. Sections were then dewaxed in xylene and hydrated through descending concentrations of alcohol (2 x 100%, 96% and 70%), rinsed in running tap water. The sections were then stained in Harris haematoxylin for 5 minutes. Excess stain was removed by rinsing in running tap water

for 5 minutes. The tap water, due to its relatively high pH, changes the haematoxylin stained elements into a deep blue colour (blueing). Sections were counterstained with-1% eosin for 3 minutes, and briefly washed in tap water. The sections were then dehydrated though ascending concentrations of ethyl alcohol (70%, 96% and 2x 100%), cleared in xylol and coversliped with DPX mountant.

Stain results: Nuclei - dark blue, Cytoplasm - bright pink

Histology of harvested foetal pancreata.

This was used to establish a light- and electron microscopic baseline for evaluating graft endocrine development against normal pancreatic development and growth.

Harvested WAG foetal pancreatic tissue of 16-18 days, 20-21 days gestation and adult WAG pancreas tissue was fixed in 10% buffered formaldehyde pH 7.2 and 2.5% phosphate buffered glutaraldehyde pH 7.4 for 18 hours, and processed for light and electron microscopy respectively.

Graft scoring.

All grafts were evaluated histologically and scored by the method described by Guymer and Mandel (1993) see Table 3 [107]. Pancreas development and survival was graded between 1 and 4. A score of 4 represented well-developed and intact grafts while a score of 1 represented severe destruction of the grafts with only secretory ducts remaining without any surviving islets. A score of 0 was awarded if no viable graft remained or had been replaced by fibrous tissue. A score of 2 was given if in addition to the secretory ducts some viable endocrine cells were

recognizable. A score of 3 was awarded if viable islets were present along with secretory ducts. Exocrine acinar tissue survival was not scored by this system as the acinar component of the graft atrophies in all foetal pancreas transplants[7,108,109]. Graft infiltration by mononuclear cells (MNC) was scored between 1 and 4. A score of 0 represented no perigraft or intragraft infiltration by mononuclear cells when viewed by high magnification (x40 objective). A score of 1 indicated only a slight perigraft infiltrate of 1 < 2 mononuclear cell per high field magnification with no intragraft MNC infiltration. A marked perigraft infiltrate together with an intragraft infiltrate of < 5 MNC/high field magnification scored 2. A score of 3 was given if there was a diffuse lymphocytic infiltrate throughout the graft with perigraft infiltration. A score of 4 represented an extensive intra- and perigraft infiltrate.

SCORE	GRAFT SURVIVAL *	GRAFT INFILTRATE
0	No survival of any tissue	No peri- or intragraft MNC infiltrates
1	Graft destroyed except for secretory ducts	Perigraft infiltrate < 2 MNC's – no intragraft infiltration
2	Secretory ducts and viable endocrine cells (loose or in groups)	A marked perigraft infiltrate with a intragraft infiltrate of < 5 MNC's
3	Secretory ducts and some viable islets of Langerhans	A diffuse infiltrate throughout the graft
4	Well developed islets of Langerhans and secretory ducts	A massive infiltrate obscuring all tissue structures

* The exocrine acinar component of the grafts was not scored

Table 3: Graft scoring after Guymer and Mandel 1993.

Immunocytochemical staining of paraffin sections [93,105]

This was used to demonstrate and classify the different endocrine cells in the islet of Langerhans by the hormones they secrete. Immunoglobulins raised against these specific peptides (hormones) allow for classification of these cells by light microscopy.

Formaldehyde fixed paraffin sections were dewaxed in xylene, hydrated through various concentrations of ethanol and washed in distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 minutes. Sections were then washed in PBS (phosphate buffered saline) pH 7.6 at room temperature. Normal goat serum (DAKO, Glostrup, Denmark, cat no X0907) diluted 1:10 with PBS was applied to all sections to block non specific Fc binding. Excess normal serum was drained from the slides and the primary antibodies applied which included, insulin (DAKO, Denmark, Glostrup, cat. no A564) diluted 1:200, glucagon (DAKO, Glostrup, Denmark cat. no A619) diluted 1:100, somatostatin (DAKO, Glostrup, Denmark cat. no A566) diluted 1: 500 and pancreatic polypeptide (DAKO, Glostrup, Denmark cat. No A619) diluted 1:1000 in PBS pH 7.6. The sections were incubated with the relevant primary antibodies for 60 minutes. Following incubation the slides were jet washed and washed in three changes of PBS of 5 minutes each. The excess buffer was drained and the biotinylated goat anti-mouse and rabbit Immunogobulin LSAB2 kit link-antibody (DAKO, Glostrup, Denmark, cat.no. K0609) applied and incubated for 30 minutes. Thereafter the sections were washed as previously described and incubated with horse rabbit peroxidase conjugated streptavidin-biotin complex (ABC) LSAB2 kit (DAKO, Glostrup, Denmark cat.no. K0609), and allowed to incubate for 30 minutes. Sections were washed as before and positive labeling demonstrated, by 0.05% 3,3"-diaminobenzidine tetrachloride (DAB) in PBS, pH 7.6, to which

hydrogen peroxide (160µl of 3%H_2O_2 was added to 50ml of the DAB solution) was added, immediately prior to incubation in the substrate for 3-5 minutes. After washing in running tap water, sections were counterstained with Mayer's Haematoxylin, dehydrated, cleared and mounted in DPX.

Results:

Positive Ig staining	brown
Background	clear
Nuclei	blue

Controls and cross reactivity

The primary antibodies selected all show a high level of inter-species cross-reactivity with rat antigens [+++ according to the DAKO inter-species cross reactivity chart (DAKO, Glostrup, Denmark)]. The DAKO LSAB2® biotinylated link antibody has been absorbed with rat, human and bovine immunoglobulins ensuring minimal cross-reactivity of the link antibody to rat tissue antigens.

Negative controls were included by substituting the primary antibody with normal mouse serum (DAKO, Glostrup, Denmark cat. no. X0910).

Electron microscopy: (method modified after Kay et al. 1967[110]).

This was used to evaluate the pancreatic grafts at an ultrastructural level. Different cell types i.e. β-cells, α-cells and δ-cells can be identified by their ultrastructural characteristics and can be

correlated with normal light microscopy and immunocytochemistry. Cell ultrastructure i.e. presence of secretory granules and other organelles, including rough endoplasmic reticulum and golgi apparatus, can also give an insight into the viability and function of cells. Early signs of "cell stress" i.e. swelling of mitrochondia and peri-nuclear space, leading to necrosis and apoptosis can be observed.

Tissue was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at 4 °C for 4 – 6 hours and cut into small blocks of no more than 1-2 mm thickness. Following fixation the tissue was rinsed in phosphate buffer to remove unbound glutaraldehyde. The tissue was post-fixed with 1.5% osmium tetroxide in Palade's buffer pH 7.4. for one hour and then rinsed with two changes distilled water to remove excess unbound osmium. Tissue was further fixed and contrasted by 2% uranyl acetate in 70% ethyl alcohol and further dehydrated in ascending concentrations of ethyl alcohol (70% for 5 minutes, 2x 96% for 5 minutes each, 3x 100% for 10, 15 and 20 minutes respectively). Following dehydration the tissue was impregnated initially with a 50/50 mixture of Spurr's resin (NSA x 13 ml, ERL 4206 x5 ml, DER x3 ml and DMAE x0.2 ml all reagents from Agar Scientific, Stansted, UK) and 100% ethyl alcohol followed by two changes of clean Spurr's resin. Resin impregnated tissue was embedded into resin filled gelatin capsules and the blocks allowed to polymerise overnight at 60°C. The tips of the capsules were trimmed with a Reichert TM60® block trimmer (Reichert, Vienna, Austria) in order to expose the embedded tissue. Semi-thin sections (1 μm) were cut on a LKB Bromma ultratome III (LKB, Vienna, Austria) with a glass knife fitted with a water trough. Sections were removed from the trough and placed on a drop of water and the sections are allowed to dry on a hot plate. Once the drop of water had evaporated and the sections adhered to the slide, the resin

was then removed from the sections with sodium methoxide. Sodium methoxide was prepared by reacting 1% metal sodium in absolute methanol. The slides were washed in tap water, to remove the sodium methoxide, and stained with warm 1% toluidine blue in 1% borax for 1 minute. Slides were washed in distilled water, blotted on filter paper and dried on a hot plate. Dry sections were mounted with DPX and studied under the light microscope. The area of particular interest was identified light microscopically and matched with the corresponding area in the resin block. The resin block was retrimmed into a small (max 2x1 mm) trapezium shape containing the area of interest by a Reichert TM60® block (Reichert, Vienna, Austria) trimmer fitted with a diamond tip trimming blade. Gold sections, thickness 90 –120 nm were cut on the LKB Bromma Ultratome III (LKB, Vienna, Austria) using the heat feeding mechanism and a glass knife with a water trough. A ribbon of sections was then picked up, from the water bath, onto a G200 copper grid and allowed to dry on a filter paper. The sections were then stained for 5 minutes with 2% acetyl acetate in 50% ethyl alcohol, rinsed in clean 50% ethyl alcohol and double stained with Reynold's lead citrate (Kay et al. 1967) for a further 5minutes. Reynold's lead citrate was prepared by mixing lead nitrate (1.33 g) with sodium citrate (1.76 g) in distilled water (30 ml). After thorough mixing the solution was allowed to react for at least one hour for the lead nitrate to convert to lead citrate. Lead citrate was dissolved with 1 N sodium hydroxide (8ml) and distilled water added to a final volume of 50 ml. After staining the grids were washed in two changes of distilled water and replaced onto a filter paper in a closed petri dish. Once the grids had dried the sections were viewed in a Hitachi H600 transmission electron microscope (Hitachi Corp, Tokyo, Japan) operated at 50 kV. Electron micrographs were taken at x1500, x3500 and x10000 to record and to analyse structures or cells.

Animals: selection, care and sample size.

Experimental design was based on close control over all variable factors influencing or skewing the outcomes of our allogeneic foetal pancreas transplant model. Breeding of donor and recipient animals was done at the Faculty of Health Sciences, University of Stellenbosch, animal breeding facility where close control was kept on atmospheric conditions and light/dark cycles. Particular attention has been paid to correlate age, sex and weight of the experimental animals. All procedures performed were standardized. Infection and spread of infection was prevented by the use of parenteral antibiotics post-transplantation and by daily cleaning and disinfection of animal cages. The animals had free access to clean drinking water and chow at all times. Taking the above into consideration together with the minimal genetic variances between the animals, thereby minimizing type I and especially type II errors, small sample groups of 5 animals were deemed sufficient per experimental group to test the hypothesis.

Controls

Both syngeneic control groups were included to assess any genetic variation within the donor groups. Provided that the strain has not become contaminated, inbred rat strains such as the WAG express a homogenous HLA halotype i.e. a major histocompatibilty complex class I (MHC class I), $RT1^u$ in the case of WAG's[6]. MHC class I mismatches have been shown to play a vital role in allograft outcome[116-120]. To test for genetic variation within our DA stock autologous transplants (DA $\Rightarrow$ DA) were included. These animals served as histological controls for non-rejecting grafts at corresponding ages. The untreated control groups were used to assess unmodified rejection in the allogeneic model. These animals will give insight as to the rate of rejection (low - or high responders) as determined histologically i.e. loss of graft tissue by

apoptosis or necrosis and infiltration of mononuclear cells into the graft. To eliminate individual lymphocytic phenotype variation, each recipient acted as its' own control for flow cytometric analysis of peripheral blood lymphocyte subsets. A specimen was taken prior to transplantation in all cases. This specimen acted as the baseline for all subsequent phenotypic values.

Statistical Analysis, Motivation and Comparison of Results.

Methods used:

Statistical analysis of the flow cytometry data of the peripheral blood lymphocyte subtypes, using the control values before each treatment, were performed by the Wilcoxon signed rank test for parametric and Mann-Whitney-U rank test for non-parametric data[116]. The Mann-Whitney ranking test for unpaired, nonparametric data was used to analyse graft survival, scored according to the method by Guymer and Mandel 1993 as described earlier[107], and to compare specific treatment groups and controls[116]. Analysis of Variation between the different treatment groups was done by the two-way ANOVA test[116]. Percentage endocrine tissue, expressed as a percentage of the total area, was used to draw up the final tables, and the data was statistically evaluated using the student's t-test for independent samples[115]. All statistical analysis was done with either Graphpad Introstat or Statistica software. A P-value of less than 0.05 ($P<0.05$) was considered as statistically significant[116].

Image analysis macro.

A macro was specifically created (Author C.J.F. Muller) to determine total islet number, islet area, immunocytochemically positive insulin and glucagon areas, which included cell numbers and cell size.

Calibration of the software

The objectives (x10, x20, x40) of the Zeiss Axiophot linked to the Image Analyser were calibrated using a 1 mm slide graticule (Nikon, Tokyo, Japan). An image of the graticule was captured with the Zeiss KS 300 and the x and y values of the image adjusted in relation to the 1mm scale on the graticule. Accuracy was confirmed by taking measurements at various lengths along the graticule scale. Once calibrated, a measurement file was saved for each objective. The appropriate measurement file was then written into the macro.

To determine islet area the macro was written to allow interactive tracing of the islet perimeter by the operator using the computer mouse. The demarcated area i.e. the total area within the traced islets was then directly calculated in μm^2 by the software.

Insulin and glucagon positive staining within the islets was differentiated from negative staining by RGB thresholding. The RGB thresholding was adjusted until only the brown staining DAB positive cells were visualized. To separate positive cells for counting, the binary image was firstly eroded and then in order, to accurately determine cell size, the image was dilated to its original size.

Data acquisition and analysis.

Acquired data of the different parameters measured were stored in a KS 300 database and exported into Excel 97 (Microsoft Corp, USA). Statistical analysis of the data was performed using either Statistica '99 (Statsoft Inc, Tulsa, USA) or Instat vers 1.15 (Graphpad Corp, USA).

Statistical Analysis of Data

Statistical analysis on data captured included total area measured, total area immunoreactive positivity for a particular hormone, percentage area of immunoreactivity in relation to total area studied[124-130].

Further calculations determined the standard error of mean, standard deviation and the percentages of the various endocrine hormones studied in relation to the total islet area.

Statistical Analysis

The p-levels reported with the test used represents the probability of error involved in assuming that a significant difference exists between and two particular series of data. P-values reported are designated "p" indicating the comparison between an internationally accepted norm and experimental values and "p1" indicating a comparison between the values of control animals and experimental values. Differences between experimental results and an accepted norm or control is considered significant if $P < 0.05$. All values of $P > 0.05$ are not considered to be significantly different [124].

BIBLIOGRAPHY

[1] Brown J, Clarck WR, Molnar IG, Mullen YS. Fetal pancreas transplantation for reversal of streptozotocin-induced diabetes in rats. Diabetes 1976; 25: 56 - 64.

[2] Brown J, Danilovs JA, Clarck WR, Mullen YS. Fetal pancreas as a donor organ. World J Surg 1984; 8: 152 - 157.

[3] Feldman SD, Scharp DW, Lacy PE, Ballinger WF. Fetal pancreas isografts, cultured and uncultured, to reverse streptozotocin- induced diabetes mellitus. Journal of Surgical Research 1980; 29: 309-318.

[4] Kemp CB, Knight MJ, Scharp DW, Balinger WF, Lacy PE. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia. 1973; 9: 486-491.

[5] Chang AY, Noble RE, Wyse BM. Streptozotocin-Induced diabetes in the chinese hamster Biochemical and Endocrine Disorders. Diabetologia 1977; 13: 595-602.

[6] Festing MFW. Inbred Strains in Biomedical Research Lab animals. 1st Ed. London: The Macmillin Press LTD; 1979.

[7] Du Toit D, Muller C, Page B, Louw J. Foetal Rat Pancreatic Transplantation: Posttransplantation Development of Foetal Pancreatic Iso- and Allografts and Suppression of Rejection With Mycophenolate Mofetil (MMF) and Cyclosporine Based immunesuppression. Microscopy Research and Technique 1998; 43 (8): 347 –355.

[8] Du Toit DF, Muller C, Mouton Y, Page B, Mattysen J, Lyners R, Woodroof C. Tacrolimus (FK506) Monotherapy Provides Potent and Significant Suppression of Allogeneic Foetal Rat pancreatic Allograft Rejection. Transplant Proc 1998; 30 (8): 4073 – 4074.

[9] Du Toit DF, Muller C, Page B, Mattysen J, Lyners R. Immunosuppression With Cyclosporin A in Combination With Mycophenolate Mofetil Suppresses Rejection of Allogeneic Fetal Rat Pancreatic Allografts. Transplant Proc 1998; 30 (8): 4092 – 4093.

[10] Muller CJF, Du Toit DF, Beyers AD, Page BJ, Muller N. Prolongation of Rat Fetal Pancreas Allograft Survival Using a Nondepleting Anti-CD4 Monoclonal Antibody W3/25. Transplant Proc 1998; 30 (8): 4180 – 4183.

[11] Muller CJF, Du Toit DF, Page BJ, Louw J, Muller BJF, Williams K, Mattysen JJ, Lyners RFC. Islet Morphology and Function Following Foetal Rat Pancreatic Transplantation. Eur J Anat, 2000; 4(3): 149 – 160.

[12] Muller CJF, Du Toit DF, Page BJ, Muller N, Mattysen J, Lyners RFC. The Efficacy of Malononitrilamide 715 (MNA) as Immunosuppressant, Alone or in Combination with Cyclosporin (CSA), in Allogeneic Foetal Rat Pancreatic Transplantion. Transplant Proc 2001; 33: 2229 – 2231.

[13] Muller CJF, Du Toit DF, Page BJ, Louw J, Muller BJF, Mattysen JJ, Lyners RFC. A Morphometric and Functional Assessment of Foetal Pancreatic Transplantation in a Syngeneic Laboratory Diabetic Rat Model. Acta Chir Austriaca 2001; 33 (174): 42.

[14] Muller CJF, Du Toit DF, Page BJ, Muller N, Mattysen J, Lyners RFC. Peri-Transplant Donor Specific Transfusion Combined With Anti-CD4 And Cyclosporine Induction Therapy Prolongs Foetal Rat Pancreas Allograft Survival. Transplant Proc 2002; 37(7): 2889 –2890.

[15] Butcher GW, Howard JC. Genetic control of transplant rejection. Transplantation. 1982; 34(4): 161-6.

[16] Stumbles P, Mason D. Activation of CD 4+ T-cells in the presence of a non-depleting monoclonal antibody to CD 4 induces a Th-2- type response in vitro. J Exp Med 1995; 182: 5-13.

[17] Valantine H. Neoral use in the cardiac transplant recipient. Transplant Proc 2000; 32(3A Suppl): 27S-44S.

[18] Dunn S. Neoral Use in the Pediatric Transplant Recipient. Transplant Proc 2000; 32 (Suppl 3A): 20S-26S.

[19] Belitsky P, Levy GA, Johnson A. Neoral Absorption Profiling: An Evolution in Effectiveness. Transplant Proc 2000; 32 (Suppl 3A): 45S-52S.

[20] Levy GA. Neoral Is Superior to FK 506 in Liver Transplantation. Transplant Proc 1998; 30: 1812-1815.

[21] Kosbashigawa JA. Controversies in Heart and Lung Transplantation Immunosuppression: Tacrolimus vs Cyclosporine. Transplant Proc 1998; 30: 1095-1097.

[22] Suthanthiran M, Strom TB. Renal transplantation. N Engl J Med. 1994; 331(6): 365-76.

[23] Kunz J, Hall MN. Cyclosporin A, FK506 and rapamycin: more than just immunosuppression. Trends Biochem Sci 1993; 18(9): 334-8.

[24] Butt TR, Graumann K, Aziz S. Molecular basis of immunosuppressive drug action: regulation of steroid receptor-mediated transcription by FK 506. Transplant Proc 1996; 28(2): 979-980.

[25] Brazelton TR, Morris RE. Molecular mechanisms of action of new xenobiotic immunosuppressive drugs: tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil and leflunomide. Curr Opin Immunol. 1996; 8(5): 710-720.

[26] Mayer AD. Four-year follow-up of the European Tacrolimus Multicenter Renal Study. Transplant Proc 1999; 31(7A): 27S-28S.

[27] Alloway RR, Russell WC, Gaber LW, Amiri MH, Vera SR, Gaber AO. Conversion from cyclosporine to tacrolimus in kidney, kidney/pancreas, and pancreas alone transplant recipients: the Memphis experience. Transplant Proc 1996; 28(2): 995-997.

[28] Peterson LB, Cryan JG, Rosa R, Martin MM, Wilusz MB, Sinclair PJ, Wong F, Parsons JN, O'Keefe SJ, Parsons WH, Wyvratt M, Sigal NH, Williamson AR, Wiederrecht GJ. A tacrolimus-related immunosuppressant with biochemical properties distinct from those of tacrolimus. Transplantation. 1998; 65(1): 10-18.

[29] Washburn WK, Shaffer D, Simpson MA, Conway P, Madras PN, Monaco AP. Tacrolimus rescue therapy for renal allograft rejection refractory to cyclosporine-based immunosuppression. Transplant Proc 1996; 28(2): 1015-1016.

[30] Ciancio G, Burke GW, Roth D, Miller J. Use of intravenous FK506 to treat acute rejection in simultaneous pancreas-kidney transplant recipients on maintenance oral FK506. Transplantation. 1997; 63(5): 785-8.

[31] Van Hooff JP, Van Duijnhoven EM, Christiaans MHL. Tacrolimus and Glucose Metabolism. Transplant Proc 1999; 31 (7): 49S-50S.

[32] Birkeland SA. Steroid-free immunosuppression after kidney transplantation with antithymocyte globulin induction and cyclosporine and mycophenolate mofetil maintenance therapy. Transplantation. 1998; 66(9): 1207-10.

[33] Qi S, Chen H, Xu D, Dalose P. Prolongation of Pancreas Allograft Survival by Mycophenolate Mofetil in the Rat. Transplant Proc 1996; 28 (2): 932 - 933.

[34] Arima T, Lehmann M, Flye MW. Induction of donor specific transplantation tolerance to cardiac allografts following treatment with non-depleting (RIB 5/2) or depleting (OX-38) anti-CD 4 MAB plus Intrathymic or intravenous donor alloantigen. Transplantation 1997; 63: 284-292.

[35] Mottram PL, Murray-Segal LJ, Han W, Zhan Y, Brady JL, Lew AM. Transgenic anti-CD4 monoclonal antibody secretion by mouse segmental pancreas allografts promotes long term survival. Transpl Immunol 2000; 8(3): 203-9.

[36] Waldmann H, Cobbold S. How do monoclonal antibodies induce tolerance? A role for infectious tolerance? Annu Rev Immunol 1998; 16: 619-44

[37] Lehmann M, Sternkopf F, Metz F, Brock J, Döcke W-D, Plantikow A, Kuttler B, Hahn H-J, Ringel B, Volk H-D. Induction of Longterm survival of rat skin allografts by novel highly efficient anti-CD 4 monoclonal antibody. Transplantation 1992; 54: 959-962.

[38] Sablinski T, Hancock WW, Tilney NL, Kupiec-Weglinski JW. CD4 monoclonal antibodies in organ transplantation--a review of progress. Transplantation 1991; 4: 579-589.

[39] Darby CR, Morris PJ, Wood KJ. Evidence that long-term cardiac allograft survival induced by anti-CD4 monoclonal antibody does not require depletion of $CD4^+$ T-cells. Transplantation 1992; 3: 483-90.

[40] Siegling A, Lehmann M, Riedel H, Platzer C, Brock J, Emmrich F, Volk HD. A nondepleting anti-rat CD4 monoclonal antibody that suppresses T helper 1-like but not T helper 2-like intragraft lymphokine secretion induces long-term survival of renal allografts. Transplantation 1994; 57(3): 464-7.

[41] Brostoff SW, Mason DW. Experimental allergic encephalomyelitis: successful treatment in vivo with a monoclonal antibody that recognizes T helper cells. J Immunol 1984; 133(4): 1938-42.

[42] Van Den Broek MF, Van De Langerijt LGM, Van Brugggen MCJ, Billingham MEJ, Van Den Berg WB. Treatment of rats with monoclonal anti-CD 4 induces Longterm resistance to Streptococal cell wall-induced arthritis. Eur J Immunol 1992; (22): 57-61.

[43] Reynolds J, Pusey CD. In vivo treatment with a monoclonal antibody to helper cells in experimental auto-immune glomerulonephritis in the BN rat. Clin. Exp. Immunol 1994; 95: 122-127.

[44] Pelegrí C, Morante MP, Castellote C, Castell M., Franch A. Administration of a non-depleting anti-CD 4 monoclonal antibody (W3/25) prevents adjuvant arthritis even upon re-challenge: parallel adminisration of a depleting anti-CD 8 monoclonal antibody (OX8) does not modify the effect of W3/25. Cellular Immunology 1995; (165): 177-182.

[45] Mannie MD, Morrison-Plummer J, Mc Connel TJ. Differentiation of Encephalitogenic T-cells Confers Resistance to an Inhibitory Anti-CD4 Monoclonal Antibody. J Immunol 1993; 151: 7293.

[46] Webb M., Mason DW, Williams A.F. Inhibition of mixed lymphocyte response by monoclonal antibody specific for rat T-lymphocyte subset. Nature 1979; 282: 841.

[47] Calne R. A Half-Century Retrospective of Transplantation as Viewd by the Proagonists: My Own Reminiscences. Transplant Proc 1999; 31: 20 - 21.

[48] Frei U. Overview of the Clinical Experience with Neoral in Transplantation. Transplant Proc 1999; 31: 1669 - 1674.

[49] Kobashigawa JA. Controversies in Heart and Lung Transplantation Immunosuppression: Tacrolimus Versus Cyclosporine. Transplant Proc 1998; 30: 1095 - 1097.

[50] Borel JF. Cyclosporine: Histotical Perspectives. Transplant Proc 1983; XV (4): 2219 - 2229.

[51] Starzl TE. The Ascension of Clinical Organ Transplantation. Transplant Proc 1999; 31: 43 - 45.

[52] Opelz G. Evaluation of Immunosuppressive Induction Regimens in Renal Transplantation. Transplant Proc 1998; 30: 4029 - 4030.

[53] Du Toit DF, Heydenrych JJ. Organ Allotransplantation since the Advent of Cyclosporin. SAMJ 1985; 68: 930 - 934.

[54] Du Toit DF, Heydenrych JJ. Siklosporien: 'n nuwe en belowende immuunonderdrukkende middel. Geneeskunde 1986; Maart: 31 - 35.

[55] Morris PJ. Cyclosporin A. Transplantation 1981; 32 (5): 349 - 354.

[56] Naji A. What's New in Transplantation. J Am Coll Surg 1997; 184: 204 - 209.

[57] Halloran, P.F. Immunosuppresive agents in clinical trials in transplantation. Am J Med Sci. 1997; 313: 283-288.

[58] Koprak S, Sirotina A, Ok H, Dumont FJ. Depletion of the mature $CD4^{+}8^{-}$ thymocyte subset by FK506 analogs correlates with their immunosuppressive and calcineurin inhibitory activities. Transplantation. 1996; 61(6): 926-32.

[59] Undre NA, Stevenson P, Schafer A. Pharmacokinetics of tacrolimus: clinically relevant aspects. Transplant Proc 1999; 31(7A): 21S-24S.

[60] Pugh-Humphreys RG, Ross CS, Thomson AW. The influence of FK-506 on the thymus: an immunophenotypic and structural analysis. Immunology. 1990; 70(3): 398-404.

[61] Keown PA. New immunosuppressive strategies. Curr Opin Nephrol Hypertens 1998; 7(6): 659-63.

[62] Knechtle SJ, Heisey DM, Pirsch JD, D'Alessandro AM, Kalayoglu M, Sollinger HW. Impact of improving immunosuppressive treatment on outcome in cadaveric kidney transplantation. Surgery 1996; 120(4): 719-24.

[63] Morris RE. Mechanisms of action of new immunosuppressive drugs. Ther Drug Monit 1995; 17(6): 564-9.

[64] Suthanthiran M, Morris RE, Strom TB. Immunosuppressants: cellular and molecular mechanisms of action. Am J Kidney Dis 1996; 28(2): 159-72.

[65] Ducloux D, Ottignon Y, Semhoun-Ducloux S, Labbe S, Saint-Hillier Y, Miguet JP, Carayon P, Chalopin JM. Mycophenolate mofetil-induced villous atrophy. Transplantation. 1998; 66(8): 1115-6.

[66] Qi S, Chen H, Xu D, Dalose P. Prolongation of Pancreas Allograft Survival by Mycophenolate Mofetil in the Rat. Transplant Proc 1996; 28 (2): 932-933.

[67] Hao L, Calcinaro F, Gill RG, Eugui EM, Allison AC, Lafferty KJ. Facilitation of specific tolerance induction in adult mice by RS-61443. Transplantation 1992; 53(3): 590-5.

[68] Hao L, Lafferty KJ, Allison AC, Eugui EM. RS-61443 allows islet al.lografting and specific tolerance induction in adult mice. Transplant Proc. 1990; 22(2): 876 - 9.

[69] Shizuru JA, Alters SE, Fathman CG. Anti-CD4 monoclonal antibodies in therapy: creation of nonclassical tolerance in the adult. Immunol Rev 1992; 129: 105-30.

[70] Auchincloss H Jr, Sultan H. Antigen processing and presentation in transplantation. Curr Opin Immunol. 1996; 8(5): 681 - 7.

[71] Fabre JW. The role of polymorphic donor peptides in allograft recognition and rejection. Immunol Rev 1996; 154: 21-43.

[72] Chen ZK, Cobbold SP, Waldmann H, Metcalfe S. Amplification of natural regulatory immune mechanisms for transplantation tolerance. Transplantation 1996; 62(9): 1200-6.

[73] Cobbold SP, Adams E, Marshall SE, Davies JD, Waldmann H. Mechanisms of peripheral tolerance and suppression induced by monoclonal antibodies to CD4 and CD8. Immunol Rev 1996; 149: 5-33.

[74] Colvin R.B. Cellular and molecular mechanisms of allograft rejection. Annu Rev Med 1990; 41: 361-375.

[75] Pietra B, Rizeq M, Bolwerk A, Gill RG. CD4 T-cells acutely reject cardiac allografts via direct donor recognition. Transplant Proc 1999; 31(1-2): 92.

[76] Onodera K, Hancock WW, Graser E, Lehmann M, Sayegh MH, Strom TB, Volk HD, Kupiec-Weglinski JW. Type 2 helper T-cell type cytokines and the development of "infectious" tolerance in rat cardiac allograft recipients. J Immunol 1997; 158(4): 1572 - 81.

[77] Strom TB, Roy-Chaudhury P, Manfro R, Zheng XX, Nickerson PW, Wood K, Bushell A. The Th-1/Th-2 paradigm and the allograft response. Curr Opin Immunol 1996; 8(5): 688-693.

[78] Sachs DH. Transplantation. Curr Opin Immunol 1996; 8(5): 671-673.

[79] Pawelec, G., Rehbein, A., Scholtz, E., Friccius, H., Pohla, H. Cytokine modulation of Th-1/ Th-2 phenotype differentiation in directly allo responsive $CD4^+$ human T-cells. Transplantation 1996; (62): 1095-1101.

[80] Takeuchi T, Lowry RP, Konieczny B. Heart allografts in murine systems. The differential activation of Th-2-like effector cells in peripheral tolerance. Transplantation 1992; 53(6): 1281-94.

[81] Liblau RS, Singer SM, McDevitt HO. Th-1 and Th-2 $CD4^+$ T-cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today 1995; 16(1): 34-38.

[82]Chen N, Gao Q, Field EH. Expansion of memory Th-2 cells over Th-1 cells in neonatal primed mice. Transplantation 1995; 60(11): 1187-93.

[83] Goedert, S., German, T., Hoehn, P., Koelsch, S., Palm, N., Rüde, E., Schmitt, E. Th-1 development of naive CD 4+ T-cells is inhibited by co-activation with anti-CD 4 monoclonal antibodies. J Immunol 1996; 157: 566-573.

[84] Kingsley CI, Karim M, Bushell AR, Wood KJ. $CD25^+CD4^+$ regulatory T-cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. J Immunol 2002; 168(3): 1080-6.

[85] Yang C-P, Sittu E, Bell EB. Specific B cell tolerance is induced by cyclosporine A plus donor-specific pretreatment: prolonged survival of MHC class I disparate cardiac allogreafts. J Immunol 2000; 164: 2427 – 2432.

[86] White RAH, Mason DW, Williams AF, Gafre G. T-lymphocyte hetreogeneity in the rat: seperation of functional subpopulations using a monoclonal antibody. J Exp Med 1978; 148: 664.

[87] Barclay AN. The localization of populations of lymphocytes defined by nomoclonal antibodies in rat lymphoid tissues. Immunology 1981; 42: 593 - 600.

[88] Webb M, Mason DW, Williams AF. Inhibition of mixed lymphocyte response by monoclonal antibody specific for rat T-lymphocyte subset. Nature 1979; 282: 841.

[89] Jefferies WA, Green JR, Williams AF. Authentic T-helper CD4 (W3/25) antigen on rat peritoneal macrophages. J Exp Med. 1985; 162(1): 117 - 127.

[90] Mason DW, Williams AF. The Kinetics of Anybody Binding to Membrane Antigens in Solution and At the Cell Surface. Biochem J 1980; 187: 1 - 20.

[91] Parham P, Androlewicz MJ, Brodsky FM, Holmes NJ, Ways JP. Monoclonal Antibodies: Purification, Fragmentation and Application to Structural and Functional Studies of Class I MHC Antigens. J Immunol Methods 1982; 52: 133 - 173.

[92] Johnstone A, Thorpe R. Immunochemistry in Practice. 3rd ed. Oxford: Blackwell Science; 1996.

[93] http://www.bdbiosciences.com/pdfs/other/BDISTechResources.pdf.

[94]Dambrin C, Klupp J, Morris RE. Pharmacodynamics of immunosuppressive drugs. Curr Opin Immunol 2000; 12(5): 557 - 62.

[95] Ilano AL, Mcconnell MV, Gurley KE, Spinelli A, Pearce NW, Hall BM. Cellular basis of allograft rejection in vivo. V. Examination of the mechanisms responsible for the differing efficacy of monoclonal antibody to $CD4^+$ T-cell subsets in low- and high-responder rat strains. J Immunol 1989; 143(9): 2828-36.

[96] Yamamoto T, Yamaguchi J, Nakayama E, Kanematsu T. Anti-CD4 induced rat heart tolerance: no presence of primed T-cells and regulatory mechanisms for cytotoxic T-cells. Transpl Immunol. 2000; 8(2): 101-7.

[97] Nikolova Z, Hof A, Baumlin Y, Hof RP. The peripheral lymphocyte count predicts graft survival in DA to Lewis heterotopic heart transplantation treated with FTY720 and SDZ RAD. Transpl Immunol. 2000; 8(2): 115-124.

[98] Francois M, Buchler M, Halimi JM, Al-Najjar A, Valentin JF, Thibault G, Lebranchu Y. Lymphocyte subsets in renal transplant recipients treated with mycophenolate mofetil. Transplant Proc 2000; 32(8): 2781-2.

[99] Thompson SC, Bowen KM, Burton RC. An assessment of flow cytometric lymphocyte subset monitoring in peripheral blood of rats as a method of detecting recipient recognition or rejection of murine endocrine xenografts. Transplantation 1988; 45(2): 489-91.

[100] Becton Dickinson Immunocytochemistry Systems (Europe). Introduction to Flow Cytometry. 1994.

[101] Darzynkiewicz Z, Crissman HA. Flow Cytometry. Methods in Cell Biology 1990; 33: 411-450.

[102] Bauer KD, Duque RE, Shankey TV, editors. Clinical Flow Cytometry: Principles and Application. Maryland: Williams & Wilkins; 1993.

[103] Coon JS, Weinstein RS, editors. Diagnostic Flow Cytometry. Baltimore: Williams & Wilkins; 1991.

[104] Herrington CS, McGee JO'D, editors. Diagnostic Molecular Pathology. A Practical Approach. Volume 1. Phenotyping and Genotyping of IntacT-cells. Oxford: IRL Press; 1992.

[105] Bancroft JD, Stevens A, editors. Theory and Practice of Histological Techniques. 3rd ed. Edinburgh: Churchill Livingstone; 1990.

[106]http://www.bdbiosciences.com/immunocytometry_systems/support/ technical_support.shtml

[107] Guymer RH, Mandel TE. Urocanic Acid as an Immunosupressant in Allotransplantation in Mice. Transplantation 1993; 55: 36.

[108] Brown J, Danilovs J.A, Clark W.R, Mullen Y.S. Fetal pancreas as a donor organ. World J Surg 1984; 8: 152-157.

[109] Eloy R, Doillon C, Dubois P. Fetal pancreatic transplantation- review of experimental data. Transplant Proc 1980; 12: 179-185.

[110] Kay D, editor. Techniques for Electron Microscopy. 2nd ed. Oxford: Blackwell Scientific Publications; 1967.

[111] Maeda A, Ito T, Ohkawa A, Tori M, Sawai T, Nozawa M, Matsuda H. Difference in immunologic responses between pancreatic and islet transplantation in "low responder" rat combinations with class I MHC disparity. Transplant Proc. 1998; 30(2): 550-1.

[112] Honjo K, Xu XY, Bucy RP. Heterogeneity of T-cell clones specific for a single indirect alloantigenic epitope (I-Ab/H-2Kd54-68) that mediate transplant rejection. Transplantation 2000; 70(10): 1516-24.

[113] Stewart R, Stephenson P, Godden U, Butcher G, Roser B. Graft rejection in a congenic panel of rats with defined immune response genes for class I antigens. II. Quantitative aspects of Ir gene function in a full-haplotype mismatch. Transplantation 1985; 40(4): 432-436.

[114] Butcher GW, Howard JC. Genetic control of transplant rejection. Transplantation 1982; 34(4): 161-166.

[115]Butcher GW, Corvalan JR, Licence DR, Howard JC. Immune response genes controlling responsiveness to major transplantation antigens. Specific major histocompatibility complex-linked defect for antibody responses to class I alloantigens. J Exp Med. 1982; 155(1): 303-320.

[116] Http:www.statsoft.com/textbook/stonlin.html

[117] McEvoy RC, Hegre OD. Morphometric quantitation of the pancreatic insulin-, glucagon-, and somatostatin-positive cell populations in normal and alloxan-diabetic rats. Diabetes 1977; 26(12): 1140-1146.

[118] Lifson N, Lassa CV, Dixit PK Relation between blood flow and morphology in islet organ of rat pancreas. Am J Physiol 1985; 249(1 Pt 1): E43-48.

[119] Rosenberg L, Duguid WP, Brown RA, Vinik AI. Induction of Nesidioblastosis will reverse diabetes in Syrian golden hamster. Diabetes 1988; 37: 334-341.

[120] Rosenberg L. Induction of IsleT-cell Neogenesis in the Adult Pancreas: The Partial Duct Obstruction Model. Microsc Res Tech 1998; 43: 337-346.

[121] Ferrand N, Astesano A, Phan H-H, Lelong C, Rosselin G, Dynamics of pancreatic cell growth and differentiation during diabetes reversion in STZ-treated newborn rats. Am J Physiol 1995; 269(5pt1): C1250 - 1264.

[122] Kramer B, Andrew A, Rawdon BB, Becker P. The effect of pancreatic mesenchyme on the differentiation of endocrine cells from gastric endoderm. Development 1987; 100(4): 661 - 671.

[123] Spandella CT, Schellini SA, Caramori CA, Bacch. Relationship Between Endocrine Function and Digital Image Quantitation of the Pancreatic Insulin- and Glucagon-Positive Cell Masses in Normal, Alloxan-Diabetic, and Heterotopic Pancreas-Transplanted Rats. Transplant Proc 2000; 32: 2820 - 2823.

[124] Page BJ. A Histological and Morphometric Assessment of Endocrine and Ductular Proliferation in the Adult Rat Pancreas using an Occlusive Pancreatic Duct Ligation Model [dissertation]. Tygerberg: University of Stellenbosch; 2000.

Chapter 7

RESULTS

Summary.

This chapter deals with the results achieved following foetal pancreatic iso- and allogeneic rat pancreas transplantation. The pharmacokinetic effect of various immnosuppressants and immunosuppression protocols (cyclosporin, mycophenolate mofetil, anti-rat CD4 monoclonal antibody (clone W3/25) and donor specfic transfusion on the peripheral blood T-cell immunophenotypes and the efficacy of these immunosuppresion protocols in preventing pancreatic allograft rejection is reflected in this chapter.

Normal Controls:

In Section one, of this chapter reference values, histological and immunocytochemical parameters of the normal adult rat are established. Peripheral blood flow cytometry reference values for T-cell immunophenotypes (CD2, CD4, CD8 and CD25) of the normal SD and PVG rats were established. The concept of gating lymphocytes from whole blood, by forward scatter vs side scatter is, introduced and the results achieved by single- and two-colour flow cytometry are reflected for comparison.

The histology and immunocytochemistry of the 17-day gestation foetal pancreas pre-transplantation and at 3-, 5-, 10-, 20- and 30-days post-transplantation are demonstrated.

Non-immunosuppressed Control Groups:

In section two, the flow cytometry and histology of the non-immunosuppressed controls used to validate the rejection potential of the DA⇒SD and DA⇒PVG allogeneic rat foetal pancreas tranplantation model, is reflected.

Cyclosporine (CsA) and Mycophenolate Mofetil (MMF) Groups:

Section three demonstrates the efficacy of cyclosporine and mycophenolate mofetil, either as monotherapies or in combination, in preventing rejection in the allogeneic foetal rat models. The pharmacokinetic effect of these conventional immunosuppressants on the peripheral blood T-cell immunophenotype is documented. Graft survival, morphology and functionality are demonstrated by histology, immunocytochemistry and electron microscopy.

Anti-CD4 (W3/25) Monotherapy Groups:

In section four, the immunosuppressive potential of a rat anti-CD4 monoclonal antibody (clone W3/25) is reflected. The dramatic effect of W3/25 therapy on the CD4 molecule is demonstrated by flow cytometry. The immunosuppressive efficacy of W3/25 as a monotherapy in preventing foetal rat allograft rejection, in the models studied, is evaluated by histology.

Donor Specific Transfusion (DST) and Combination Therapy Groups:

In section five, results of immunosuppressive protocols, in which donor specific transfusion (DST) were used either alone or in combination with CsA and W3/25 induction therapy, are presented. In this section the combination of DST with 5-days of CsA and W3/25 induction resulted in graft acceptance and development without the need for daily immunosuppression. Flow cytometry was used to establish the effect that the different and combined therapies had on the PBL T-cell immunophenotypes. Harvested grafts were histologically evaluated and graft survival determined. Immunocytochemistry and electronmicroscopy on the DST, CsA and W3/25 combination therapy groups demonstrated the efficacy of the protocol in preventing graft rejection and resulting in the development of morphologically normal and functional islets.

Autologous and Allogeneic Diabetic Groups and Controls:

In section six, the efficacy of iso- and allogeneic foetal rat transplantation in the reversal of streptozotocin-induced diabetes is demonstrated. Metabolic profiles and glucose tolerance test results, comparing normal and non-transplanted diabetic controls, are compared to the transplanted diabetic animals. The harvested grafts were evaluated by histology, immunocytochemistry and electron microscopy. Morphometry of the islets of Langerhans was performed to establish islets size and cell composition.

Histological Graft Scoring – All Foetal Rat Pancreas Transplantation (FRPT) Groups:

In the final section (seven) of this chapter, graft score for all the experimental groups are tabulated. The scoring method, as described by Guymer and Mandel (1991), was used to enumerate graft survival and graft infiltration by mononuclear cells.

Results Achieved - Conclusions:

1. Isografts display near normal islet histology (endocrine/ICC).

2. No immunosuppression (across major histocompatibilty barrier – DA ⇒ SD and DA ⇒ PVG) results in unmodified rejection within 4 –9 days.

3. Summarised histological results (DA ⇒ SD and DA ⇒ PVG):

Groups	Treatment	Histology
CsA	5 mg/kg/d	Good graft survival
MMF	50 mg/kg/d	Heavy graft infiltration – rejection
CsA+MMF	5 mg/kg/d+50 mg/kg/d	Excellent graft survival
W3/25	200 μg/d	Graft survival + heavy infiltrate
W3/25	500 μg/d	Good graft survival + light infiltrate
DST	3 ml	Complete rejection
DST + CsA	3 ml + 5 mg/kg/d/5 days	Rejection (some graft survival – PVG)
DST + W3/25	3 ml + 500 μg/d/5 days	Graft survival + heavy infiltrate
DST + CsA + W3/25	3 ml + 5 mg/kg/d/5 days+ 500 μg/d/5 days	Excellent graft survival + light infiltrate

4. Summarised flow cytometry results (DA ⇒ SD and DA ⇒ PVG):

Groups	Treatment	Pharmacokinetic effect on T-cells
CsA	5 mg/kg/d	$CD2^+CD4^+$ slight decline; p=NS
MMF	50 mg/kg/d	No effect
CsA+MMF	5 mg/kg/d+50 mg/kg/d	$CD2^+CD4^+$ decline; p=0.001
W3/25	200 µg/d	Rapid $CD2^+CD4^+$ decline; p=0.008
W3/25	500 µg/d	Rapid $CD2^+CD4^+$ decline; p=0.008
DST	3 ml	No effect
DST + CsA	3 ml + 5 mg/kg/d/5 days	$CD2^+CD4^+$ mariginal effect; p=NS
DST + W3/25	3 ml + 500 µg/d/5 days	Rapid $CD2^+CD4^+$ decline; p=0.05
DST + CsA +	3 ml + 5 mg/kg/d/5 days	SD rapid $CD2^+CD4^+$ decline; p=0.008

Functional:

5. Isogeneic FRPT can render diabetic rats normoglycaemic

6. Allografts without immunesuppression are unable to induce normoglycaemia in diabetic rats due to rapid rejection of the grafts.

7. DST, CsA and W3/25 induction therapy results in allograft unresponsiveness that allows for graft growth and development, without daily immunosuppression, capable of inducing normoglycaemia in diabetic rats.

SECTION 1.

NORMAL CONTROLS AND ISOGENEIC RAT FOETAL PANCREAS CONTROLS.

1.1. Results of flow cytometry used to establish normal reference ranges are reflected in Figures 19a, 19b and 20a - 20F and in tables 4 and 5.

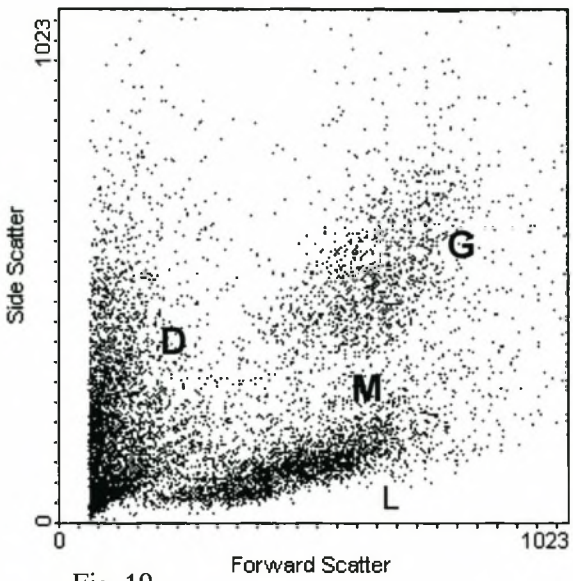

Fig. 19a.

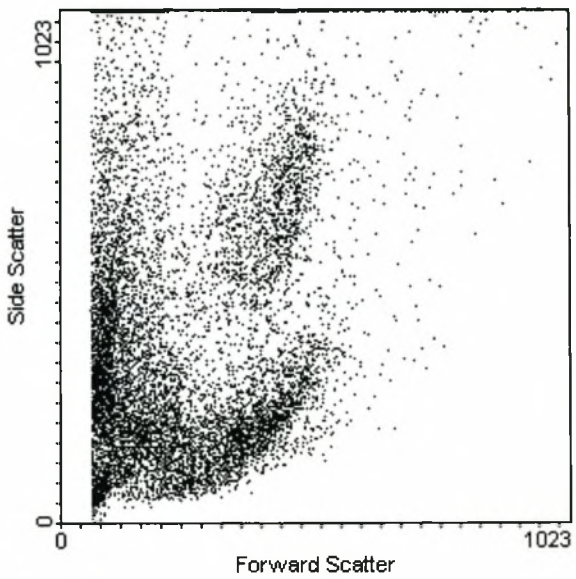

Fig. 19b.

Figure 19a shows forward vs. side scatter dot plot which was used to distinguish the lymphocyte populations (L) other leukocytes (monocytes M and granulocytes G) and debris (D) by their relative positions on the dot plot. Figure 19b demonstrates a poor quality dot plot that makes accurate discrimination of populations impossible. Attempting to gate on indistinct lymphocyte populations, which inevitably includes unlysed red blood cells and debris in the gate, affects the accuracy of the researcher's analysis.

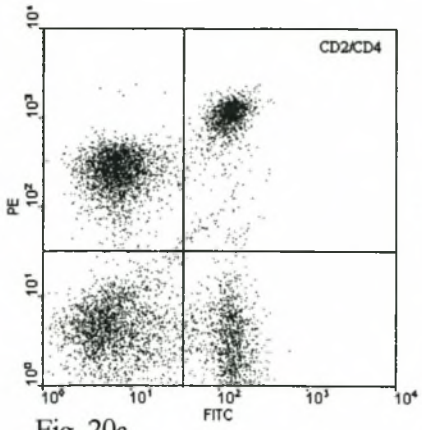

Fig. 20a.

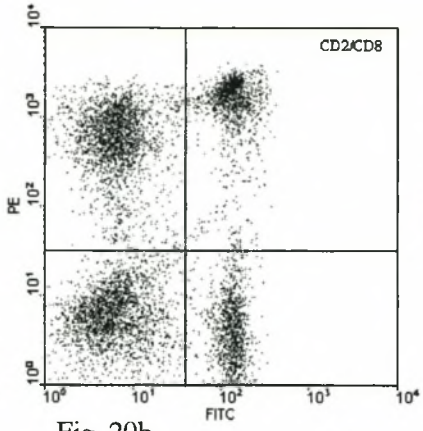

Fig. 20b.

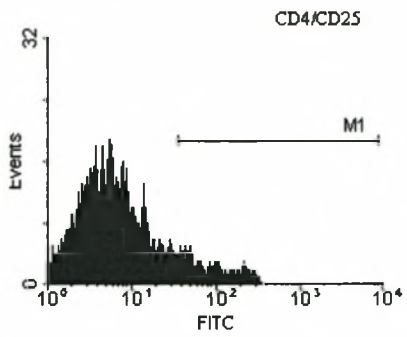

Fig. 20c.

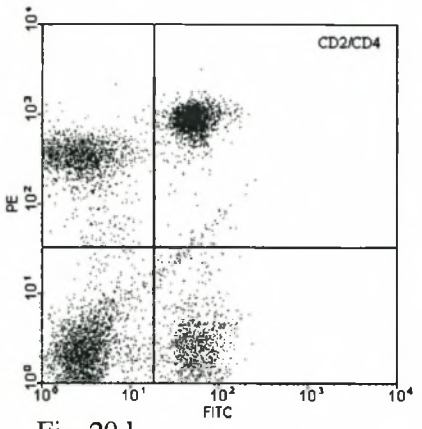

Fig. 20d.

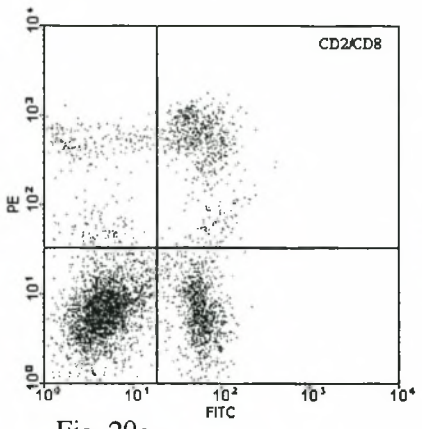

Fig. 20e.

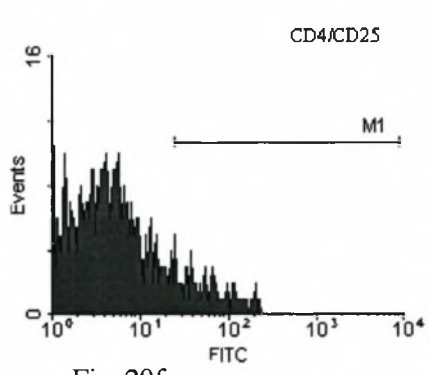

Fig. 20f.

Figures 20a, 20b and 20c show the typical flow cytometric dot plots and histogram used to determine the peripheral blood lymphocyte (PBL) immunophenotypic profiles of the mature male SD rat. Within a FSC vs SSC lymphocyte gate the mean PBL percentage for CD2+CD4+ lymphocytes was 51.5% ± 1.6 and 48.7% ± 1.8 for CD2+CD8+ lymphocytes. The CD4+CD25+ mean PBL percentage was 5.2 ± 0.5. The PBL immunophenotypic profile of the mature male PVG rat as represented by the dotplots (Figures 20d, 20e) and histogram 5.2 ± 0.5 (figure 20f) showed a mean percentage of CD2+CD4+ lymphocytes to be 72.0% ± 2.0, the CD2+CD8+ lymphocytes were 24.3% ± 1.3 and CD4+CD25+ PBL's were 7.2% ± 0.8.

Strain	CD2	CD4	CD2/CD4	CD8	CD2/CD8	CD4/CD8	CD4/CD25
SD	48.52	35.08	72.3	12.37	25.5	1.38	19.58
SD	48.79	33.18	68	18.16	37.22	0.89	3.87
SD	45.62	31.16	68.3	9.8	21.5	1.45	N/A
SD	40.7	26.66	65.5	13.46	33.07	0.81	28.22
SD	41.46	27.32	65.9	10.3	24.8	1.10	20.24
SD	45.62	33.49	73.4	8.97	19.7	1.70	N/A
SD	35.7	24.6	68.9	9.39	26.3	0.94	13.94
SD	42.94	26.45	61.6	10.4	24.2	1.09	4.76
SD	33.56	22.72	67.7	16.05	47.8	0.48	6.3
SD	43.52	30.25	69.5	7.97	18.3	1.65	7.64
SD	44.36	27.81	62.7	14.8	33.4	0.83	1.86
Mean	42.8 ± 1.4	28.9 ± 1.2	67.6 ± 1.1	12.0 ± 1.0	28.3 ± 2.7	1.1 ± 0.1	11.8 ± 3.0
PVG	58.6	45.8	78.2	10.08	17.2	2.66	3.2
PVG	41.17	30.27	73.5	6.92	16.8	1.80	2.37
PVG	29.5	21.87	74.4	5.58	18.9	1.16	1.32
PVG	30.62	22.03	71.9	5.48	17.9	1.23	3.71
PVG	41.27	29.83	72.28	6.81	16.5	1.81	22.21
PVG	48.15	30.82	64	7.8	16.2	1.90	28.62
PVG	39.93	29.94	74.98	6.27	15.7	1.91	12.62
PVG	44.68	31.04	69.47	6.39	14.3	2.17	7.78
PVG	39.71	30.26	76.2	6	15.1	2.00	23.2
Mean	41.51 ± 2.9	30.21 ± 2.3	72.77 ± 1.4	6.81 ± 0.5	16.51 ± 0.5	1.85 ± 0.2	11.67 ± 3.5

Table 4: Single colour flow cytometric PBL analysis of untreated normal control rats (unmodified immunophenotypic profiles)

Strain	CD2	CD4	CD2/CD4	CD8	CD2/CD8	CD4/CD8	CD4/CD25
SD	48.38	23.2	47.9	24.89	51.5	0.93	4.28
SD	32.9	17.22	52.1	15.35	46.9	1.11	5.62
SD	30.7	17.5	57.4	14.02	45.4	1.26	4.56
SD	21.63	10.34	46.6	12.33	58.5	0.80	3.46
SD	37.08	19.47	51.3	17.48	48.3	1.06	4.33
SD	39.71	24.07	59.4	16.26	41.8	1.42	6.04
SD	41.95	20.11	49.4	19.57	45.3	1.09	5.84
Mean	36.6 ± 2.9	18.9 ± 1.5	51.5 ± 1.6	17.6 ± 1.4	48.7 ± 1.8	1.1 ± 0.1	5.2 ± 0.5
PVG	41.34	31.16	75.4	9.76	23.6	3.19	6.83
PVG	47.37	31.07	65.4	13.65	28.9	2.26	12.09
PVG	41.2	32.09	76.2	8.7	21.6	3.53	5.68
PVG	32.85	24.53	75.3	6.29	19	3.96	5.92
PVG	34.69	26.66	73.9	6.59	19.8	3.73	6.84
PVG	40.35	29.85	72.6	8.07	20.4	3.56	7.01
PVG	55.51	34.67	63.4	13.86	24.6	2.56	6.3
PVG	46.77	33.86	74.11	13.36	27.92	2.65	N/A
PVG	34.17	24.9	71.68	10.9	32.44	2.21	N/A
Mean	41.6 ± 2.9	29.9 ± 1.3	72.0 ± 2.0	10.1 ± 1.2	24.3 ± 1.3	3.1 ± 0.2	7.2 ± 0.8

Table 5: Two colour flow cytometric PBL immunophenotype analysis of untreated normal control rats (unmodified immunophenotypic profiles).

1.2. Histology and immunocytochemistry of the normal mature SD pancreas and islet of Langerhans are reflected in Figures 21 to 25.

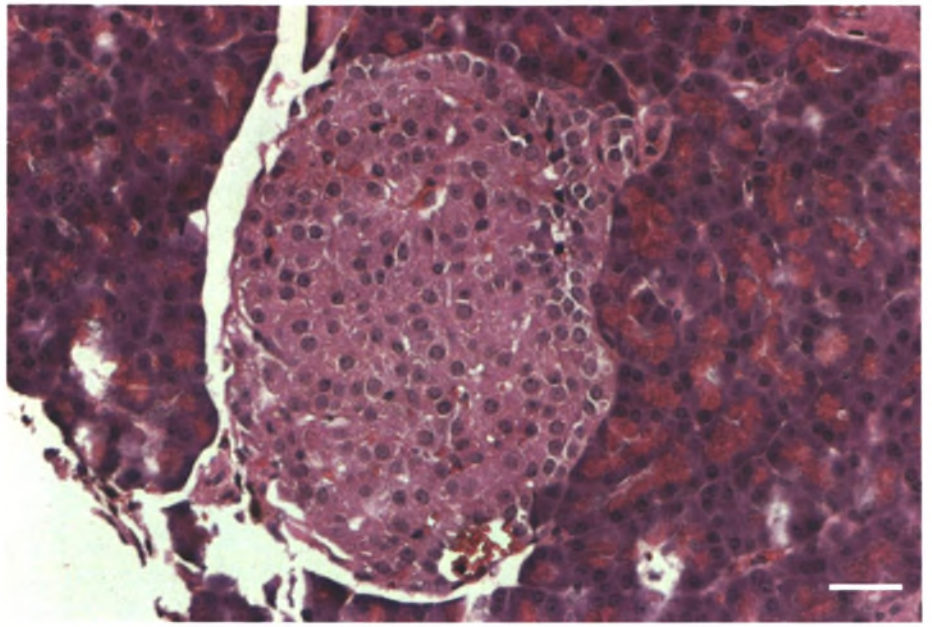

Figure 21: Normal pancreas of the SD shows the presence of a large islet of Langerhans surrounded by numerous serous acini that make up the largest part of the exocrine portion of the pancreas (H&E x 400). Scale bar = 20 μm.

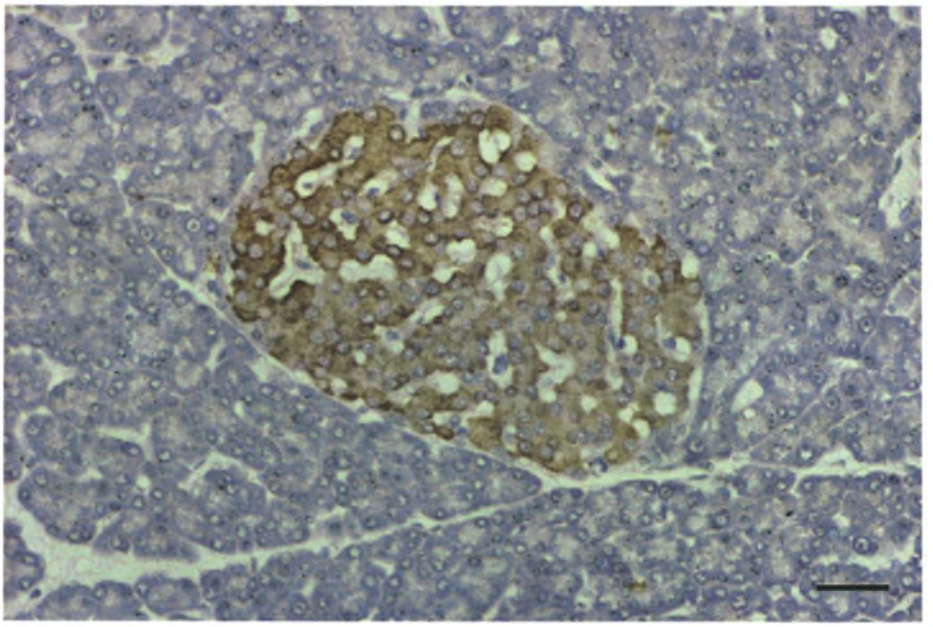

Figure 22: Immunocytochemistry demonstrating that the islet of Langerhans consists mainly of insulin positive (brown staining) β-cells, which make up approximately 60% of the islets area in the normal pancreas (insulin x400). Scale bar = 20 μm.

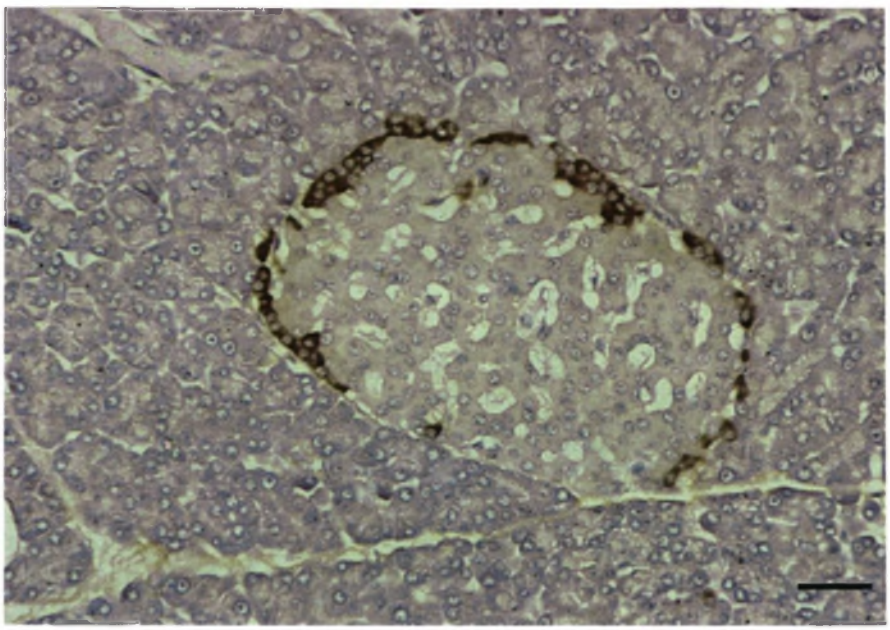

Figure 23: Glucagon positive α-cells are less numerous than β-cells constituting up to 20% of the islet of Langerhans area. They are mostly limited to the peripheral part of the islet (glucagon x400). Scale bar = 20 μm.

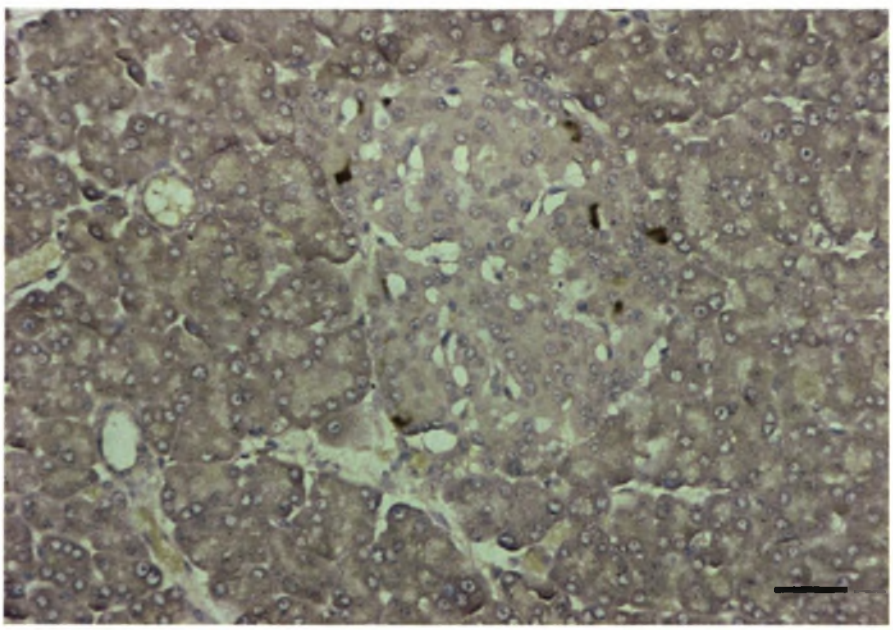

Figure 24: Immunocytochemistry demonstrating somatostatin shows the presence of a few single δ-cells that are found in the periphery of the islet (somatostatin x400). Scale bar = 20 μm.

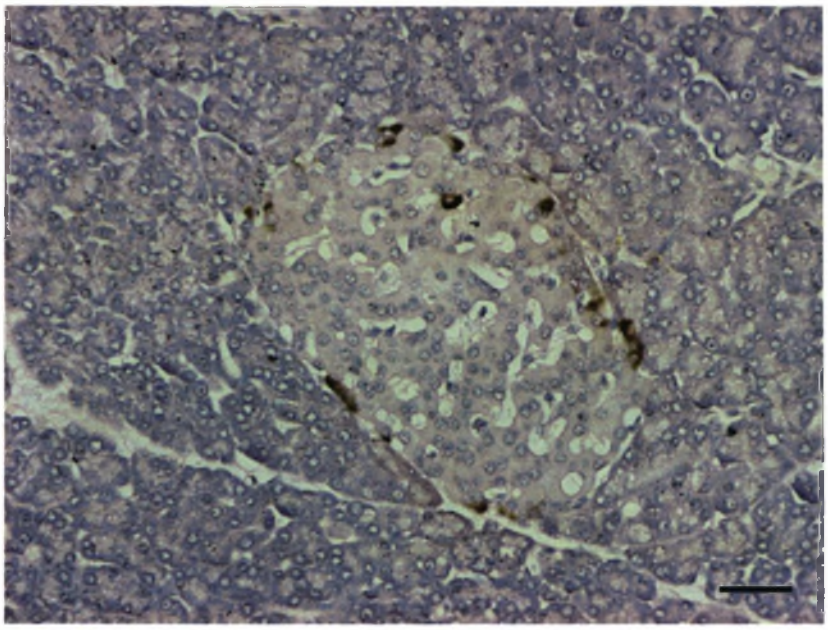

Figure 25: Pancreatic polypeptide positive F-cells are also seen as single cells limited to the periphery of the islet (PP x400). Scale bar = 20 μm.

1.3. Histology, immunocytochemistry and electron microscopy of the 17-day gestation foetal DA pancreas are reflected in Figures 26 to 30.

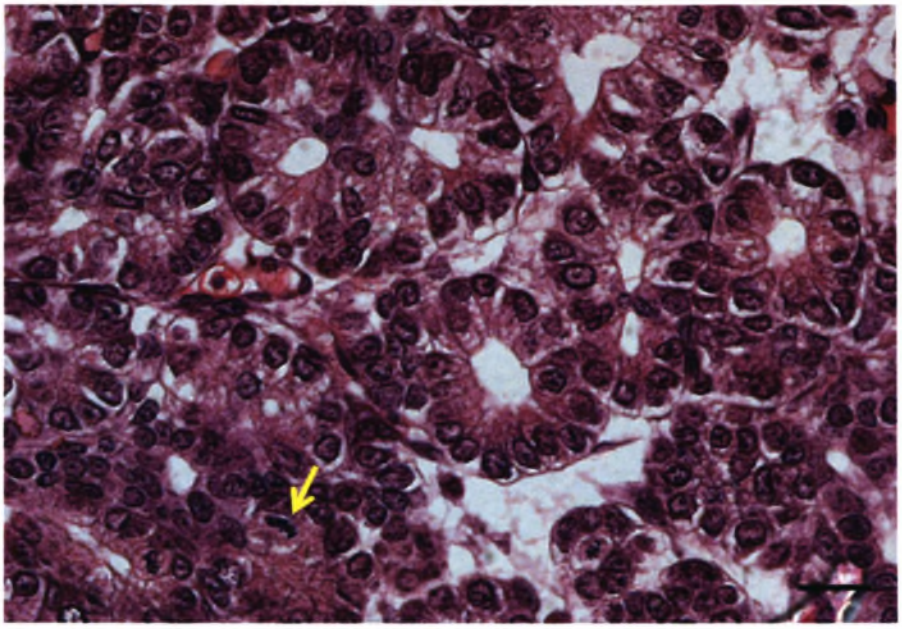

Figure 26: The typical histology of a 17 day gestation foetal pancreas. The pancreas consists of undifferentiated ductular structures and capillaries. Note the mitotic figure (arrow) (H&E x 400). Scale bar = 20 μm.

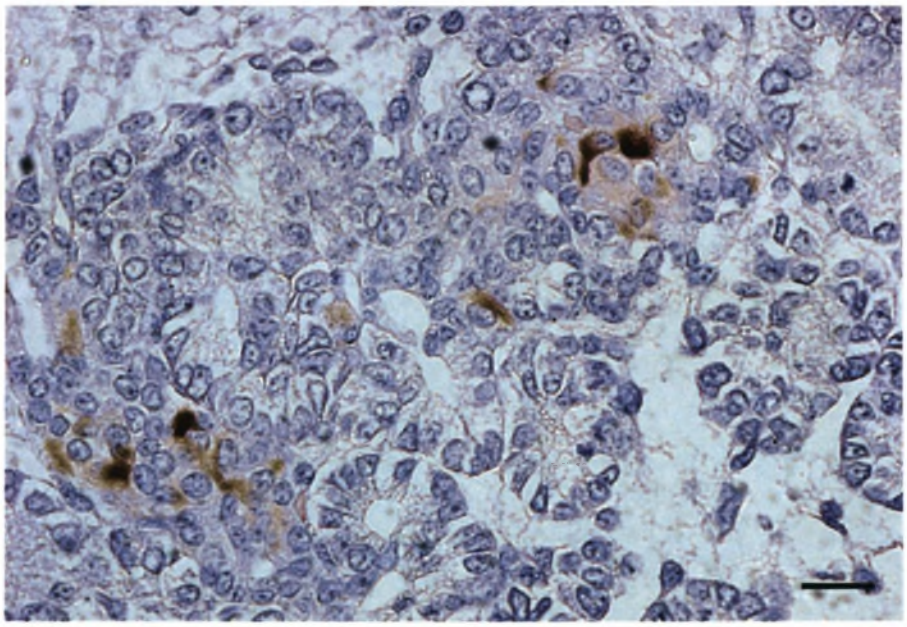

Figure 27: Insulin positive staining cells are seen as single cells or loosely arranged small clusters associated with ductules. No islets are recognised in the 17 day gestation control foetal pancreas (insulin x400). Scale bar = 20 μm.

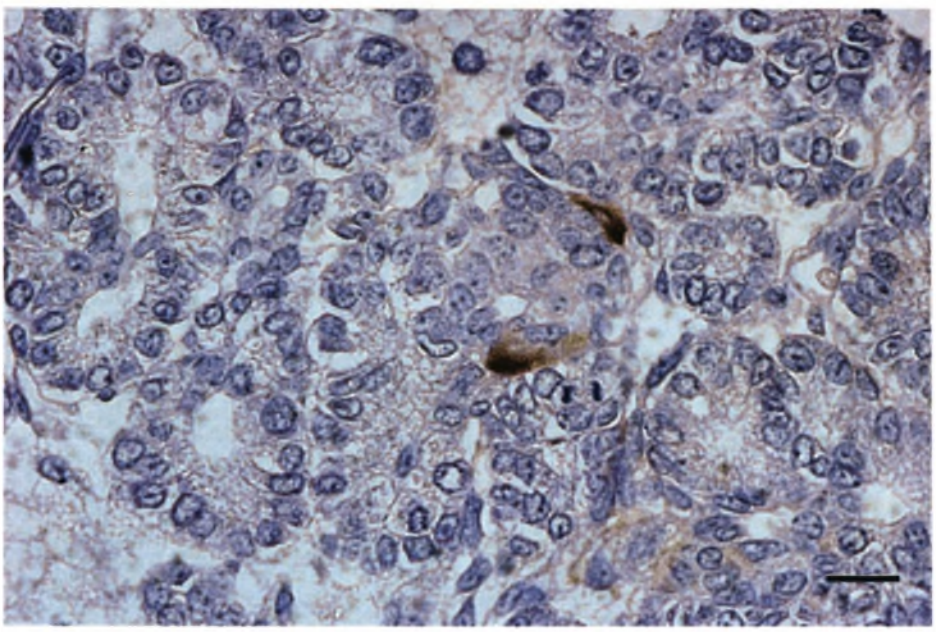

Figure 28: Glucagon positive staining cells are present as single cells associated with the ductules in the 17 day gestation control foetal pancreas (glucagon x 400). Scale bar = 20 μm.

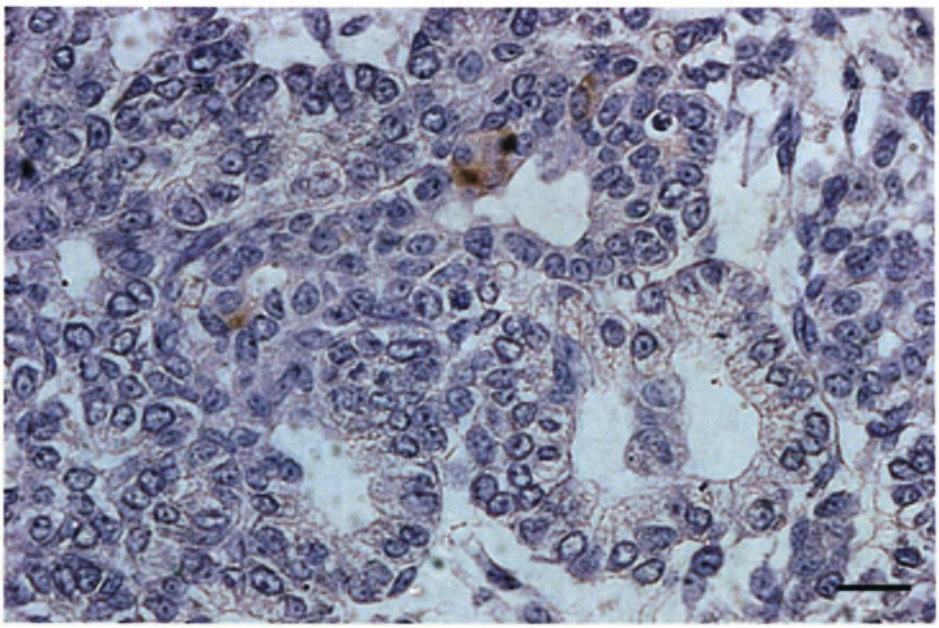

Figure 29: Somatostatin positive staining cells were rarely seen in the 17 day gestation pancreas but when present they appeared as single cells associated with the ductules (anti-somatostatin x 400). Scale bar = 20 μm.

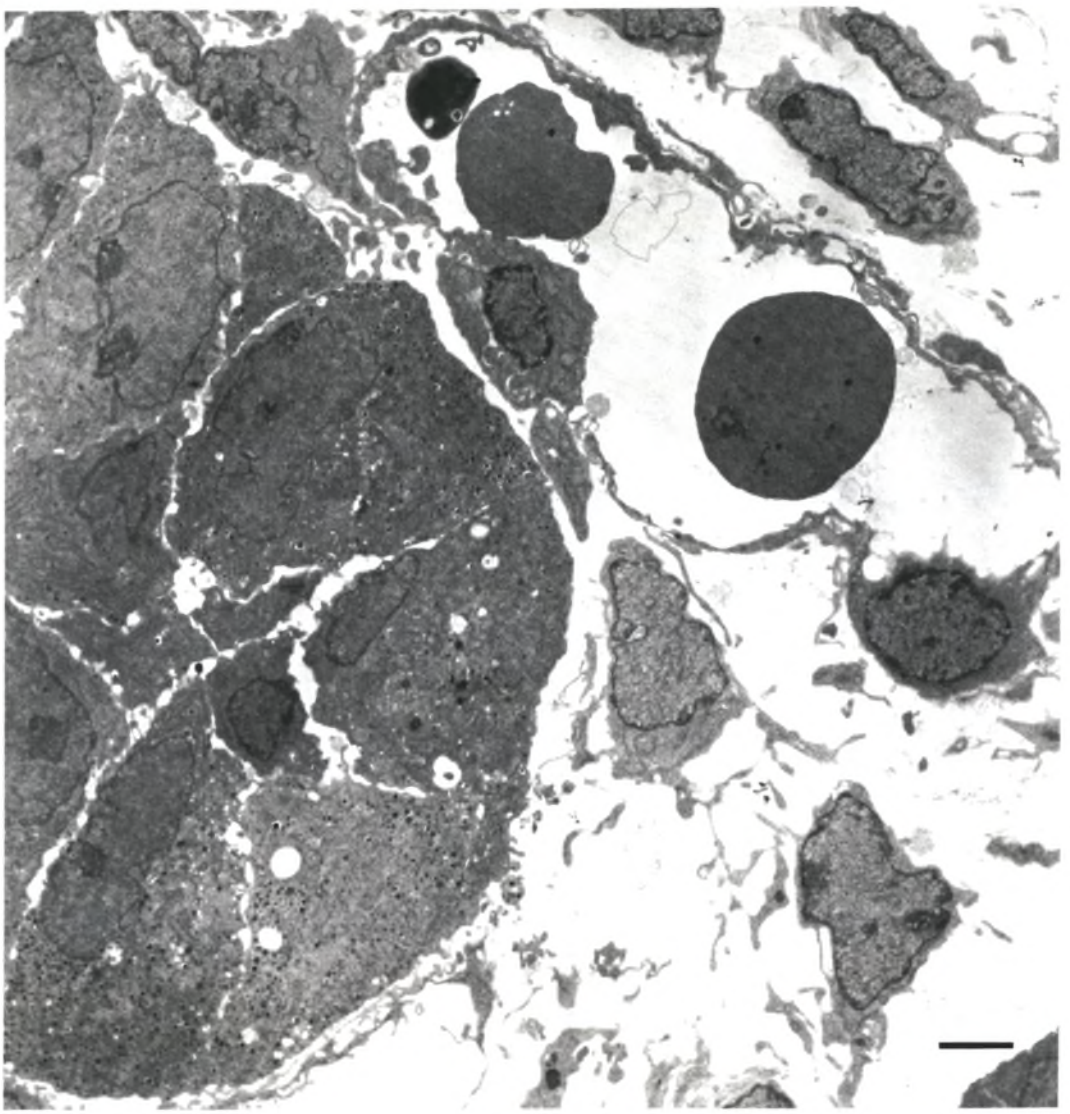

Figure 30: Electronmicrograph of a 17 day gestation foetal pancreas showing a ductule containing granulated endocrine cells with the ultrastructural features of α- and β-cells. The interstitium contains undifferentiated mesenchymal cells and capillaries (x4500). Scale bar = 2.2 μm.

1.4. The immunocytochemistry of transplanted 17 day gestation DA foetal rat pancreas isografts harvested at days 3, 5, 10, 20 and 30 are reflected in Figures 31 to 42.

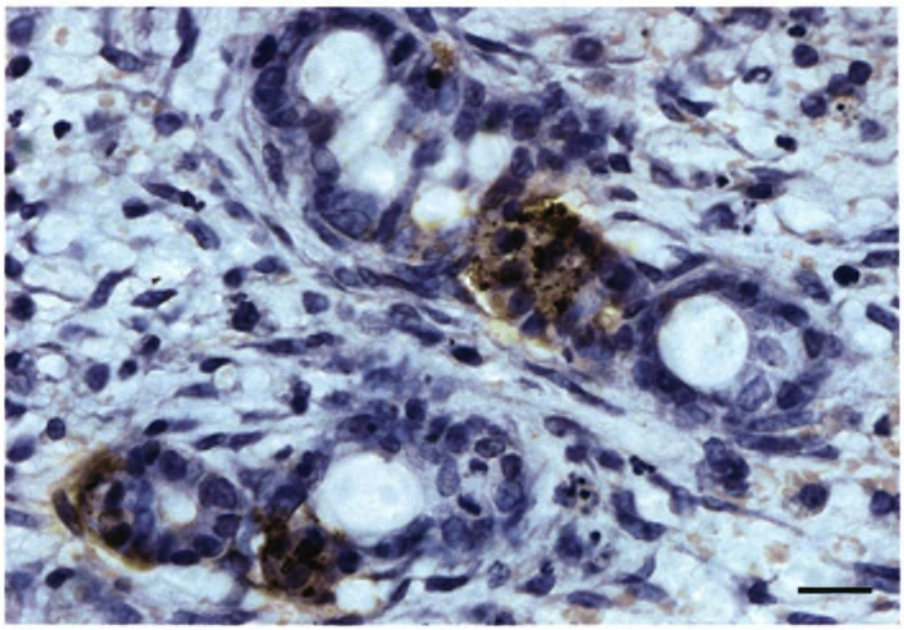

Figure 31: Insulin staining of a foetal rat pancreas isograft, 3 days post-transplantation, shows the development of small clusters of positive staining cells. In all cases the clusters are in close association with the ductules (insulin x 400). Scale bar = 20 μm.

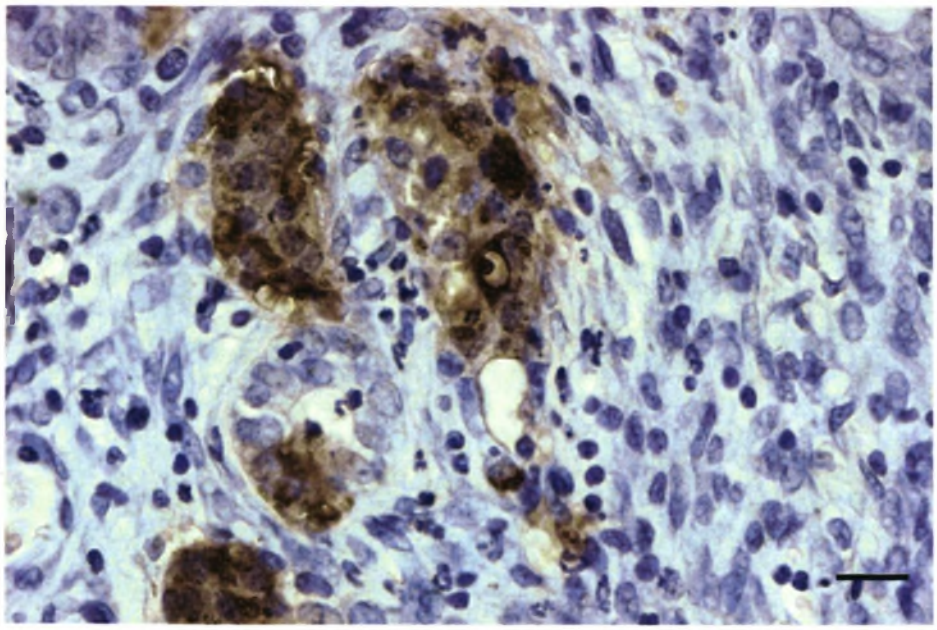

Figure 32: Insulin staining of the foetal pancreatic isografts, 5 days post-transplantation, showed clusters of positive cells either in association with ductules or loose in the surrounding stroma. These clusters appear to be more organised with the morphological appearance of small islets. Note mitotic cell in islet (insulin x 400). Scale bar = 20 μm.

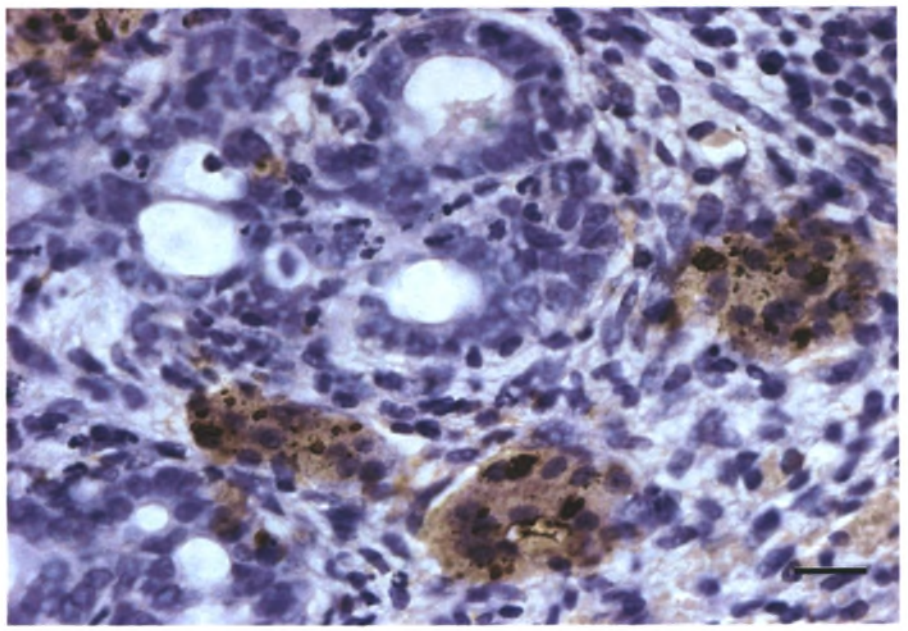

Figure 33: 10 days post-transplantation: small insulin positive islets appear in the stroma. These islets are mostly not associated with the remaining ductules (insulin x 400). Scale bar = 20 μm.

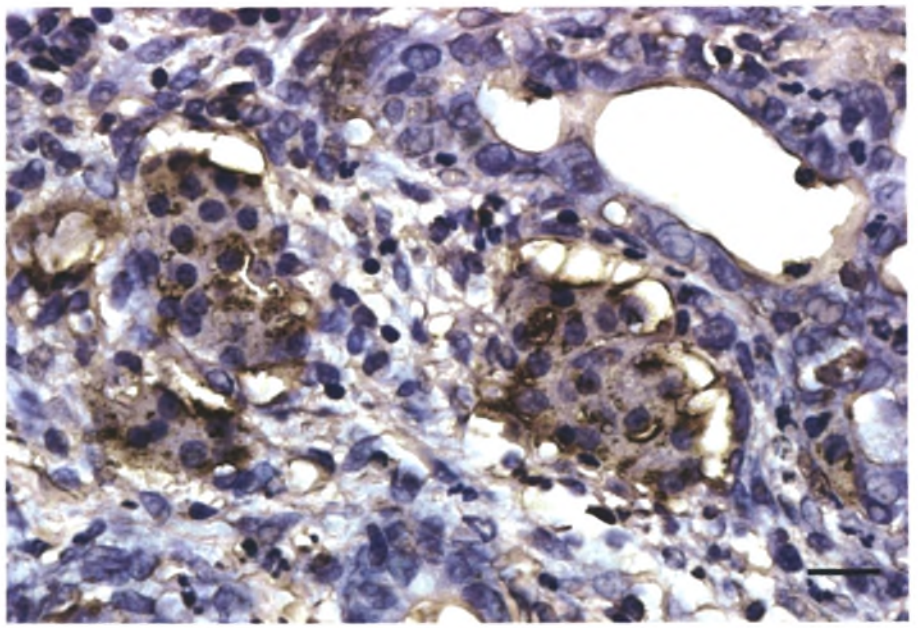

Figure 34: At 20 days post-transplantation: the islets consist of more mature and larger polygonal cells. The remaining ductules show signs of atrophy with excessive widening of the lumens (insulin x 400). Scale bar = 20 μm.

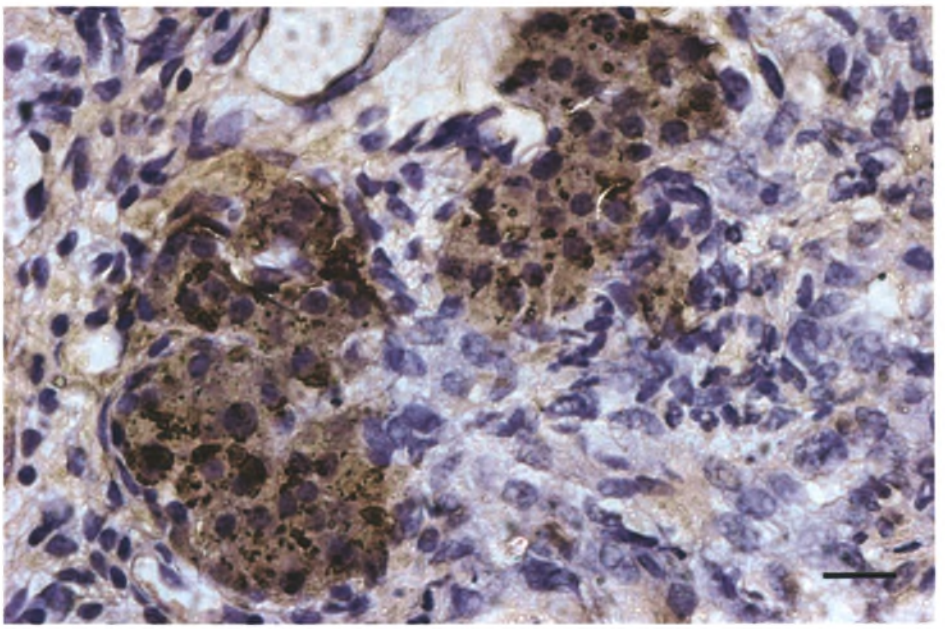

Figure 35: 30 days post-transplantation: the isografts show the appearance of mature islets consisting almost entirely of insulin positive cells within fibrous tissue (insulin x 400). Scale bar = 20 μm.

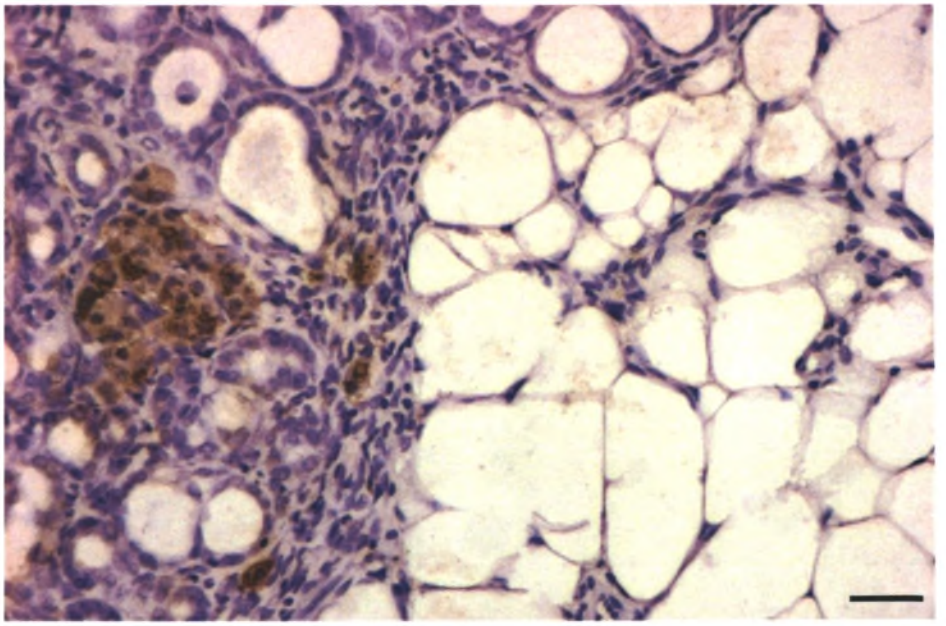

Figure 36: A FRP isograft at 30 days post-transplantation shows the close association between the islets and adipose tissue. Ductules and other exocrine graft components have undergone extensive atrophy (insulin x 400). Scale bar = 20 μm.

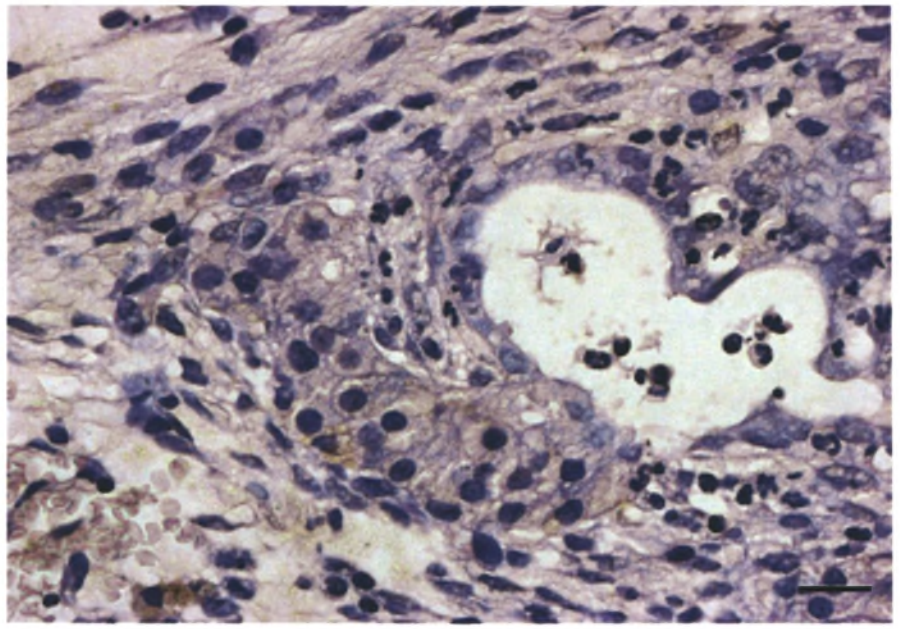

Figure 37: Glucagon positive cells are visible and appear as single lightly staining cells 10 days post-transplantation (glucagon x 400). Scale bar = 20 μm.

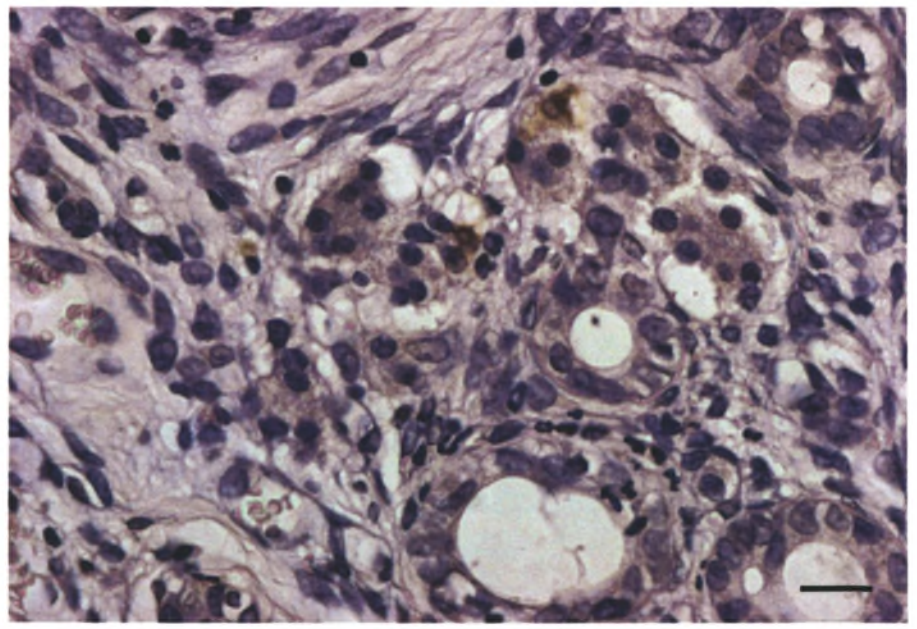

Figure 38: By 20 days post-transplantation single glucagon positive cells are seen associated with small islets (glucagon x 400). Scale bar = 20 μm.

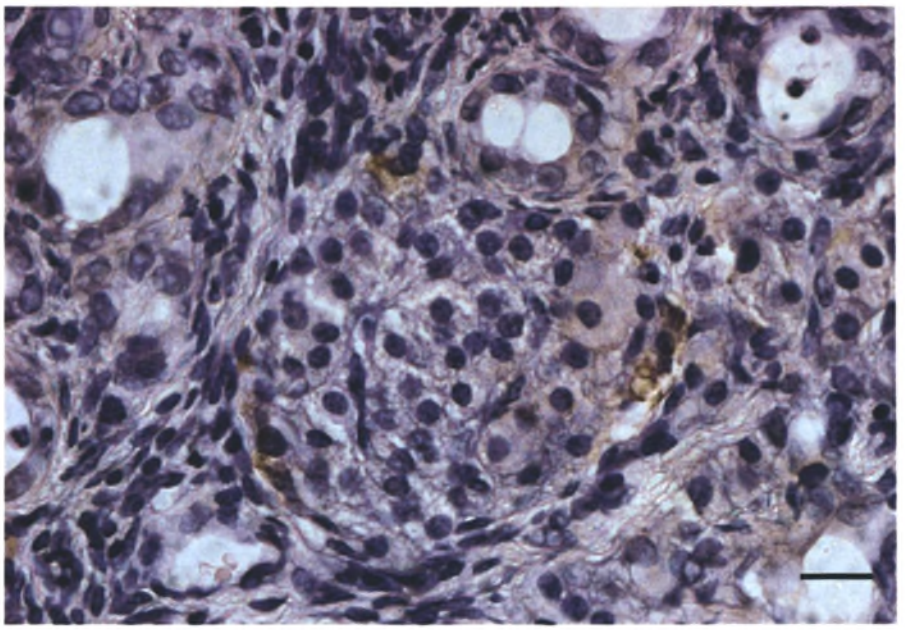

Figure 39: 30 days post-transplantation: glucagon positive cells appear as single cells in the mantle zone of the isogeneic islets (glucagon x 400). Scale bar = 20 μm.

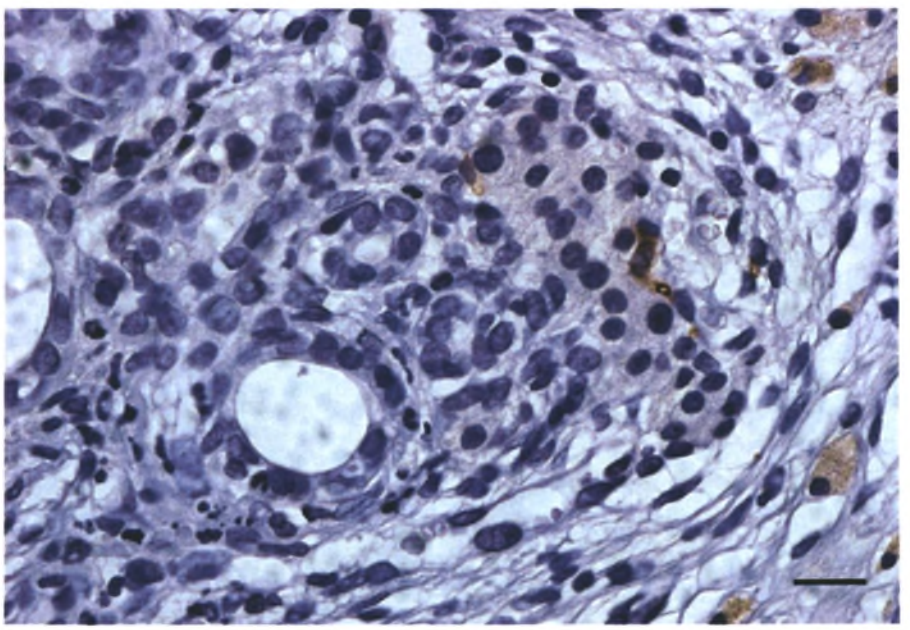

Figure 40: Somatostatin positive cells first appear around 10 days post-transplantation. Somatostatin positive cells are rare appearing as single cells (somatostatin x 400). Scale bar = 20 μm.

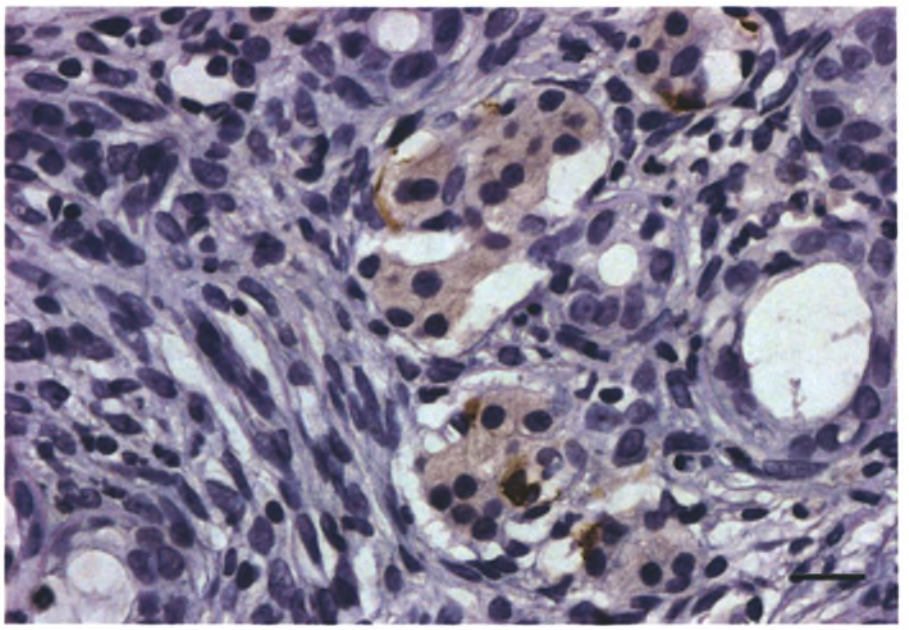

Figure 41: At 20 days post-transplantation single somatostatin positive cells are seen in the peripheral part of the isogeneic islets (somatostatin x 400). Scale bar = 20 μm.

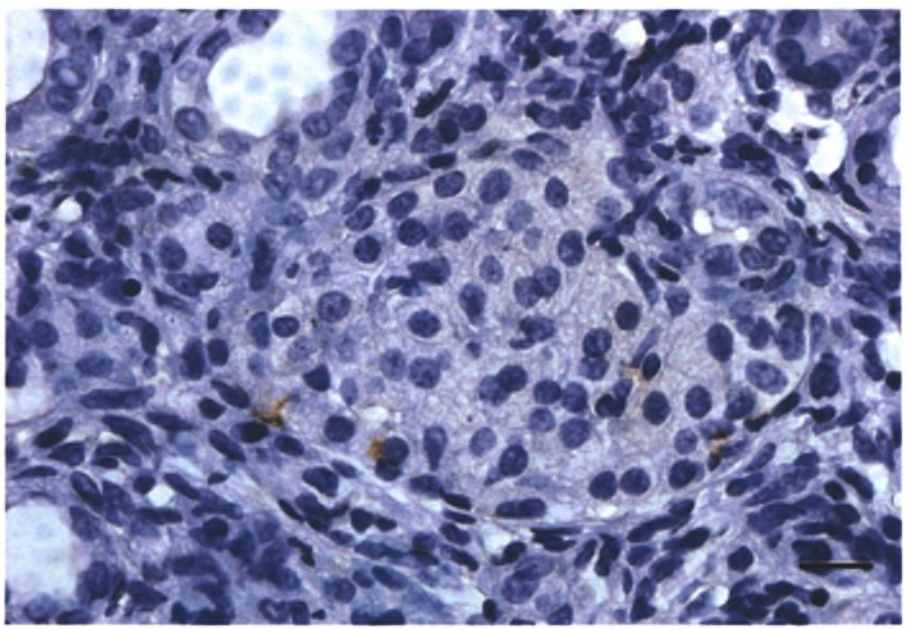

Figure 42: By 30 days post-transplantation the occasional somatostatin positive cells appear as single cells in the mantle zone of the islets (somatostatin x 400). Scale bar = 20 μm.

SECTION 2.

RESULTS TRANSPLANTATION CONTROL GROUPS – UNMODIFIED REJECTION.

The unmodified rejection groups were included to establish the rejection potential of the two transplantation models.

2.1. Results of flow cytometry used to establish whether the transplantation procedure, the introduction of allogeneic tissue, antibiotics had an effect on the T-cell immunophenotypes are shown tables 6 and 7.

Whole Blood Lymphocyte Flow Cytometry Non-immunosuppressed Controls

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	43.2 ± 0.6	54.7 ± 0.9	44.4 ± 0.4	1.1 ± 0.02	6.0 ± 0.2
Day 1	38.4 ± 1.1 (p=0.2)	52.7 ± 1.1 (p=0.1)	47.9 ± 0.5 (p=0.07)	1.1 ± 0.03 (p=0.1)	6.0 ± 0.3 (p=0.9)
Day 7	40.4 ± 1.5 (p=0.4)	54.2 ± 4.3 (p=0.5)	45.2 ± 1.8 (p=0.3)	1.2 ± 0.1 (p=0.1)	5.2 ± 0.2 (p=0.5)
Day 14	42.5 ± 0.6 (p=0.5)	53.3 ± 1.8 (p=0.4)	43.8 ± 2.5 (p=0.5)	1.3 ± 0.1 (p=0.1)	7.7 ± 0.8 (p=0.3)

Table 6: Group 3 - Two colour flow cytometric PBL analysis of transplantation controls (DA ⇒SD) without immunosuppression (unmodified rejection profiles).

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	41.6 ± 0.8	73.1 ± 0.5	21.6 ± 1.3	3.4 ± 0.2	5.6 ± 0.2
Day 1	37.6 ± 1.3 (p=0.2)	70.1 ± 1.0 (p=0.3)	25.7 ± 0.6 (p=0.2)	2.7 ± 0.1 (p=0.1)	6.3 ± 0.4 (p=0.5)
Day 7	40.1 ± 0.7 (p=0.7)	72.6 ± 0.7 (p=0.5)	24.8 ± 0.7 (p=0.3)	2.9 ± 0.1 (p=0.2)	7.0 ± 0.7 (p=0.09)
Day 14	41.6 ± 0.7 (p=0.8)	72.9 ± 0.8 (p=0.5)	24.4 ± 0.7 (p=0.2)	3.0 ± 0.1 (p=0.4)	6.7 ± 0.3 (p=0.1)

Table 7: Group 16: Two colour flow cytometric PBL analysis of transplantation controls (DA ⇒PVG) without immunosuppression (unmodified rejection profiles).

2.2. Histological assessment of the grafts that had been completely rejected are reflected in Figures 43 to 44.

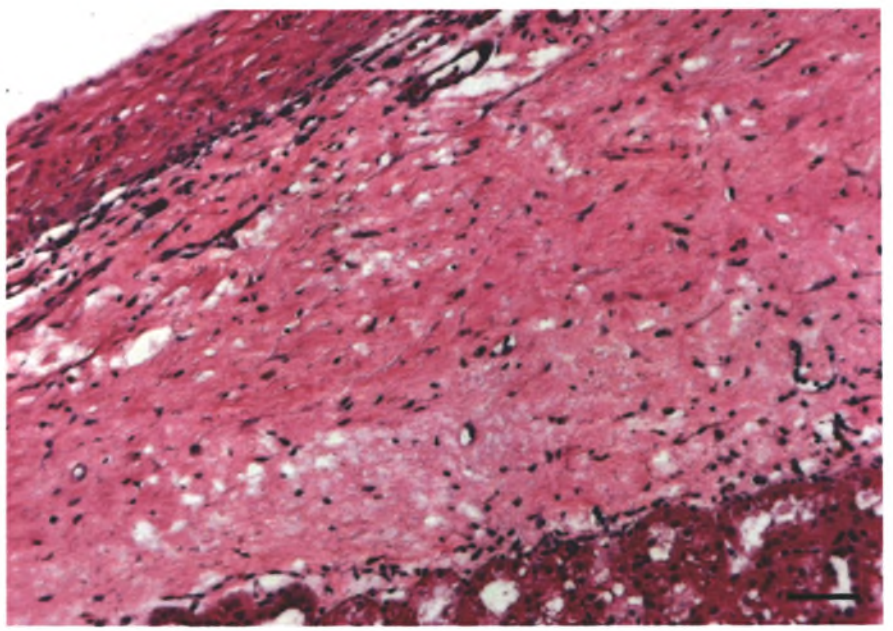

Figure 43: Group 3 - Histology showed severe rejection of FRPT in the DA to SD allogeneic model. By 14 days post-transplantation the grafts have disappeared and have been completely replaced by fibrous tissue. Note the presence of the overlying kidney capsule, the underlying

kidney parenchyma, and the mononuclear infiltrate at the graft kidney interface (H&E x 100). Scale bar = 90 μm.

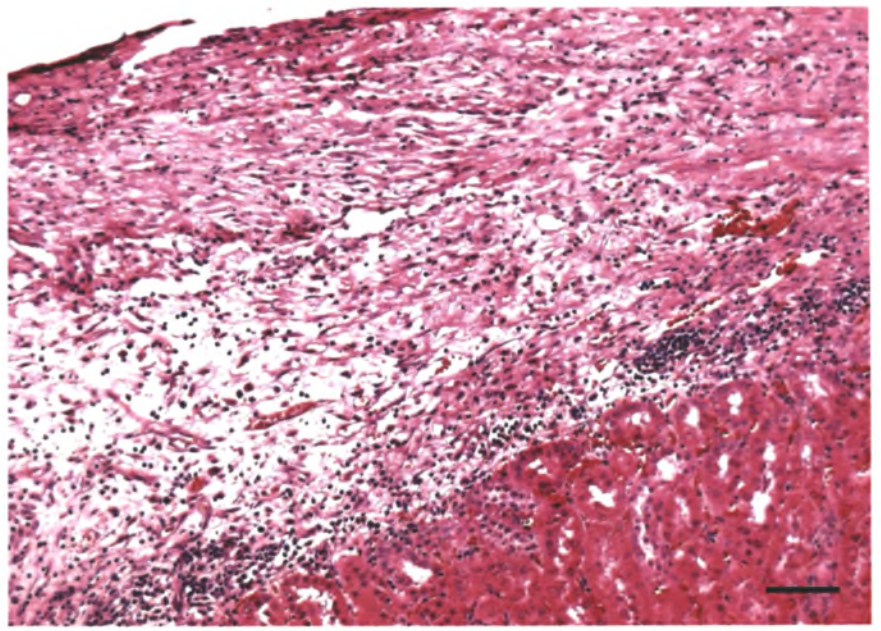

Figure 44: Group 16 - The untreated controls (transplantation across a strong histocompatibility barrier without immunesuppression) following FRPT in the DA to PVG allogeneic model also showed advanced and acute rejection 14 days post-transplantation. A band of mononuclear cells consisting mostly of lymphocytes can be seen at the graft kidney interface (H&E x 100). Scale bar = 90 μm.

SECTION 3.

Results of the CsA, MMF and CsA/MMF combination immunesuppression Transplantation Groups.

CsA and MMF are well-established immunosuppressants used widely in transplantation. CsA is still regarded as the mainstay in most immunosuppression regimes and its mechanism of action is well known. MMF, a purine synthesis inhibitor, further enhances the efficacy of CsA in the clinical setting. This combination can be regarded as the gold standard in terms of the prevention of graft rejection in the rat pancreas transplantation model.

3.1. The pharmacokinetic effect of CsA, MMF and CsA/MMF combination therapies on the T-cell immunophenotypes, studied flow cytometrically, are reflected in Figure 45 and in Tables 8 and 13.

Whole Blood Lymphocyte Flow Cytometry of the CsA, MMF and CsA/MMF combination groups.

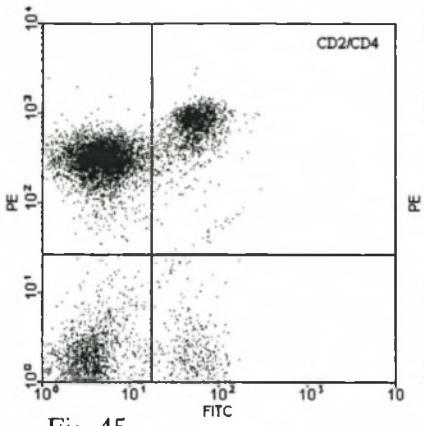

Fig. 45a.

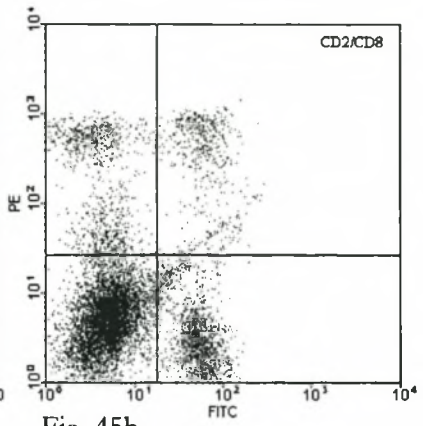

Fig. 45b.

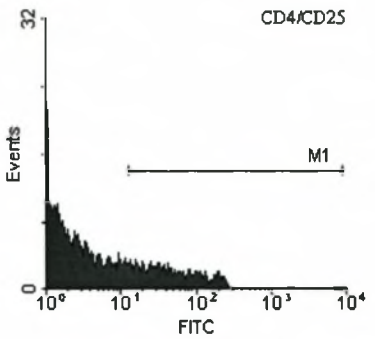

Fig. 45c.

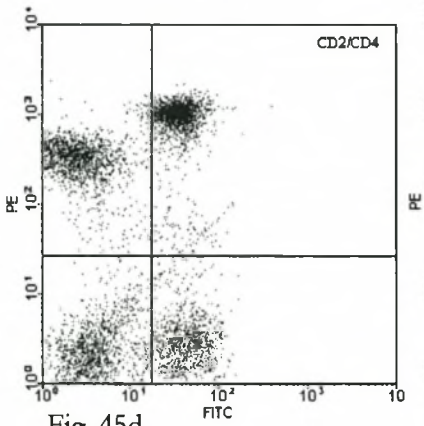

Fig. 45d.

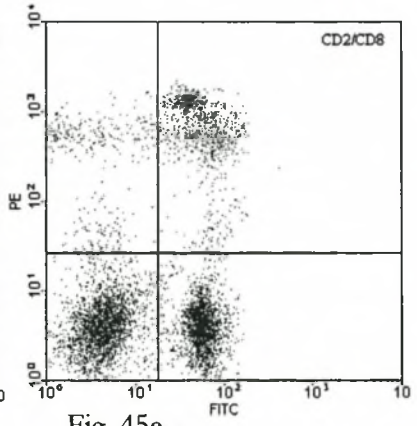

Fig. 45e.

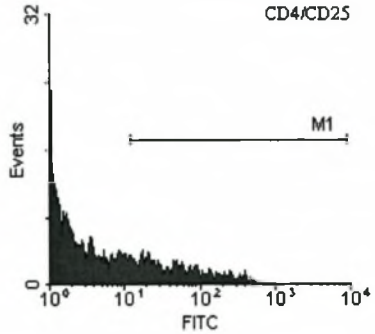

Fig. 45f.

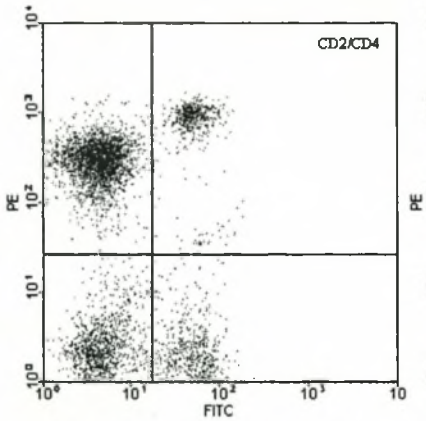

Fig. 45g.

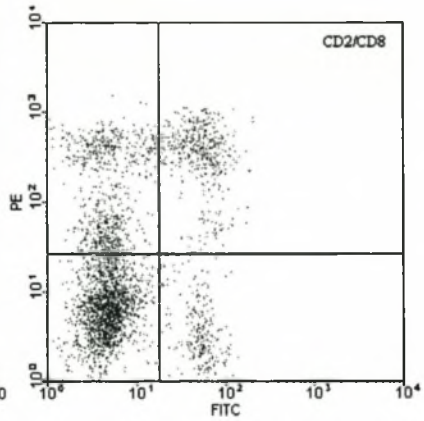

Fig. 45h.

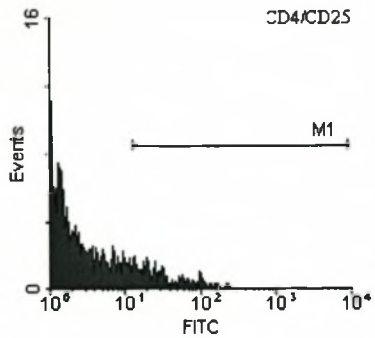

Fig. 45i.

Figure 45: CsA monotherapy at the given dose of 5 mg/kg/d (group 17) had a small, but not significant (p=0.08) , suppressive effect on the $CD2^+CD4^+$ PBL profile of the adult PVG rat (Figures 45a, 45b and 45c). MMF monotherapy at the given dose of 50 mg/kg/d (group 18) had no measurable effect on the T-cell PBL's (figs 45d, 45e and 45f). In combination however CsA and MMF had a general suppressive effect on the $CD2^+CD4^+$ T-cell PBL's (p=0.001). Daily doses of CsA (5 mg/kg/d) and MMF (50mg/kg/d) resulted in a gradual but significant decline of $CD2^+CD4^+$ percentage in the adult male PVG rat from 71.3 ± 1.2 to 62.1 ± 2.0 and 52.4 ± 1.7 at 14 and 30 days post-transplantation respectively (Figs 45g, 45h and 45i).

Day Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	40.0 ± 1.2	52 ± 0.8	47.7 ± 0.8	1.1 ± 0.1	6.1 ± 0.1
Day 1	27.8 ± 0.5 (p=0.008)	46.8 ± 1.1 (p=0.004)	50.4 ± 0.9 (p=0.05)	0.9 ± 0.1 (p=0.01)	6.5 ± 0.2 (p=0.3)
Day 7	39.9 ± 0.9 (p=0.8)	48.3 ± 0.6 (p=0.08)	48.3 ± 1.0 (p=0.5)	1.0 ± 0.1 (p=0.08)	6.5 ± 0.2 (p=0.2)
Day 14	40.2 ± 0.6 (p=0.8)	48.7 ± 0.8 (p=0.08)	47.9 ± 0.4 (p=0.8)	1.0 ± 0.1 (p=0.08)	6.3 ± 0.3 (p=0.8)

Table 8: Group 4 - Two colour flow cytometric PBL analysis following CsA 5mg/kg/d monotherapy (DA ⇒ SD).

Day Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	41.5 ± 0.9	70.5 ± 0.9	26.8 ± 1.1	2.5 ± 0.07	6.5 ± 0.3
Day 1	35.0 ± 1.5 (p=0.1)	67.5 ± 1.2 (p=0.06)	29.0 ± 1.0 (p=0.2)	2.3 ± 0.09 (p=0.3)	7.0 ± 0.2 (p=0.2)
Day 7	37.5 ± 1.1 (p=0.2)	48.3 ± 0.6 (p=0.005)	28.2 ± 0.9 (p=0.4)	1.0 ± 0.03 (p=0.004)	6.5 ± 0.2 (p=0.2)
Day 14	37.2 ± 0.8 (p=0.1)	67.4 ± 1.4 (p=0.08)	28.3 ± 0.2 (p=0.5)	2.4 ± 0.05 (p=0.5)	7.1 ± 0.2 (p=0.08)

Table 9: Group17 – Two-colour flow cytometric PBL analysis following CsA 5 mg/kg/d monotherapy (DA ⇒ PVG).

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	39.8 ± 1.2	51.4 ± 0.9	47.3 ± 1.2	1.1 ± 0.02	6.3 ± 0.2
Day 1	37.5 ± 1.3 (p=0.2)	50.1 ± 1.4 (p=0.3)	48.9 ± 1.8 (p=0.3)	1.1 ± 0.03 (p=0.8)	6.8 ± 0.5 (p=0.3)
Day 7	36.8 ± 1.8 (p=0.1)	49.4 ± 2.0 (p=0.1)	48.9 ± 2.3 (p=0.3)	1.0 ± 0.02 (p=0.1)	6.2 ± 0.9 (p=0.8)
Day 14	34.2 ± 1.3 (p=0.07)	50.2 ± 2.0 (p=0.4)	48.3 ± 1.9 (p=0.3)	1.0 ± 0.03 (p=0.1)	6.5 ± 0.6 (p=0.8)

Table 10: Group 5 - Two-colour flow cytometric PBL analysis following MMF monotherapy 50 mg/kg (DA ⇒SD).

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	43.8 ± 1.0	70.7 ± 1.0	28.1 ± 1.3	2.5 ± 0.08	7.2 ± 0.5
Day 1	37.3 ± 3.2 (p=0.03)	68.2 ± 1.3 (p=0.2)	27.9 ± 1.5 (p=0.8)	2.4 ± 0.1 (p=0.1)	7.0 ± 0.5 (p=0.8)
Day 7	41.6 ± 1.6 (p=0.8)	68.8 ± 1.1 (p=0.2)	30.0 ± 0.9 (p=0.08)	2.3 ± 0.07 (p=0.1)	6.8 ± 0.4 (p=0.3)
Day 14	39.2 ± 0.9 (p=0.08)	67.2 ± 1.3 (p=0.1)	31.7 ± 1.4 (p=0.08)	2.1 ± 0.2 (p=0.08)	6.7 ± 0.6 (p=0.3)

Table11: Group 18 - Two-colour flow cytometric PBL analysis following MMF monotherapy (DA ⇒PVG).

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	41.3 ± 1.7	53.1 ± 1.5	48.1 ± 1.1	1.1 ± 0.1	6.0 ± 0.7
Day 1	39.8 ± 1.8 (p=0.4)	50.3 ± 2.1 (p=0.1)	48.9 ± 1.5 (p=0.4)	1.0 ± 0.1 (p=0.4)	5.7 ± 0.6 (p=0.4)
Day 7	39.7 ± 2.0 (p=0.4)	50.8 ± 1.8 (p=0.1)	50.1 ± 1.3 (p=0.2)	1.0 ± 0.1 (p=0.3)	5.2 ± 1.2 (p=0.3)
Day 14	38.2 ± 2.6 (p=0.4)	44.9 ± 2.3 (p=0.001)	52.7 ± 2.0 (p=0.001)	0.9 ± 0.1 (p=0.001)	6.0 ± 1.6 (p=0.8)
Day 30	35.3 ±2.7 (p=0.08)	41.4 ± 2.0 (p=0.001)	54.1 ± 1.7 (p=0.001)	0.8± 0.1 (p=0.001)	5.6 ± 0.9 (p=0.4)

Table 12: Groups 6 and 7 - Two-colour flow cytometric PBL analysis following CsA and MMF combination therapy (DA ⇒SD).

Day Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	41.5 ± 2.9	71.68 ± 1.4	32.44 ± 1.0	2.2 ± 0.1	5.8 ± 1.3
Day 1	32.3 ± 1.8 (p=0.1)	67.6 ± 1.2 (p=0.1)	33.8 ± 1.9 (p=0.4)	2.0 ± 0.09 (p=0.4)	5.5 ± 0.5 (p=0.8)
Day 7	38.9 ± 2.3 (p=0.1)	65.7 ± 1.3 (p=0.1)	31.2 ± 1.3 (p=0.4)	2.1 ± 0.1 (p=0.4)	6.1 ± 0.9 (p=0.3)
Day 14	41.7 ± 1.7 (p=0.1)	61.6 ± 1.6 (p=0.0003)	33.2 ± 0.9 (p=0.0001)	1.9 ± 0.1 (p=0.0001)	5.8 ± 0.4 (p=0.8)
Day 30	38.0 ± 1.1 (p=0.3)	57.0 ± 1.9 (p=0.001)	34.2 ± 1.5 (p=0.001)	1.7 ± 0.1 (p=0.001)	5.8 ± 0.4 (p=0.8)

Table 13: Groups 19 and 20 - Two-colour flow cytometric PBL analysis following CsA and MMF combination therapy (DA ⇒PVG).

3.2. Histological assessment of the grafts in CsA, MMF and CsA/MMF combination groups are reflected in Figures 46 to 53.

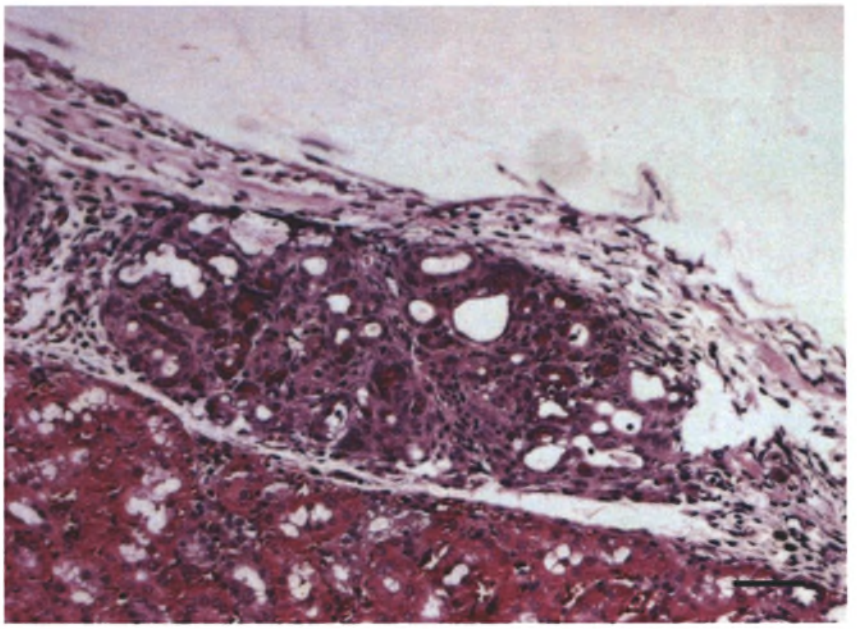

Figure 46 - Group 4: Cyclosporine monotherapy 5 mg/kg/d prevented graft rejection. A well-preserved FRP allograft is clearly visible beneath the kidney capsule. Very little graft infiltrate is present (H&E x 100). Scale bar = 90 μm.

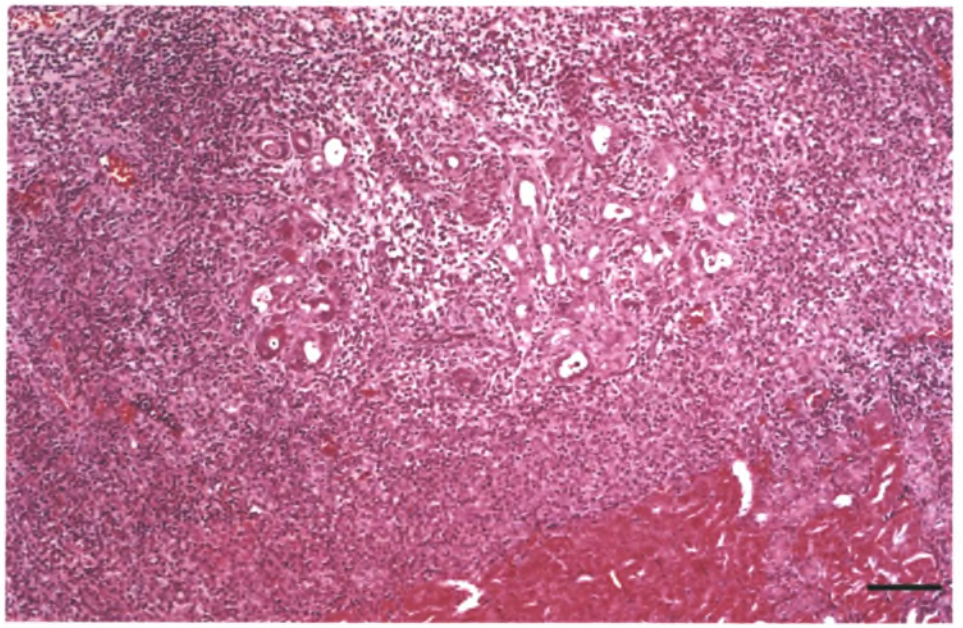

Figure 47 - Group 5. MMF monotherapy (oral 50 mg/kg/d) failed to prevent a massive influx of mononuclear cells into the graft resulting in acute, irreversible rejection. Some remaining graft, mostly dilated ducts and islets still survive within the heavy infiltrate (H&E x50). Scale bar = 170 μm.

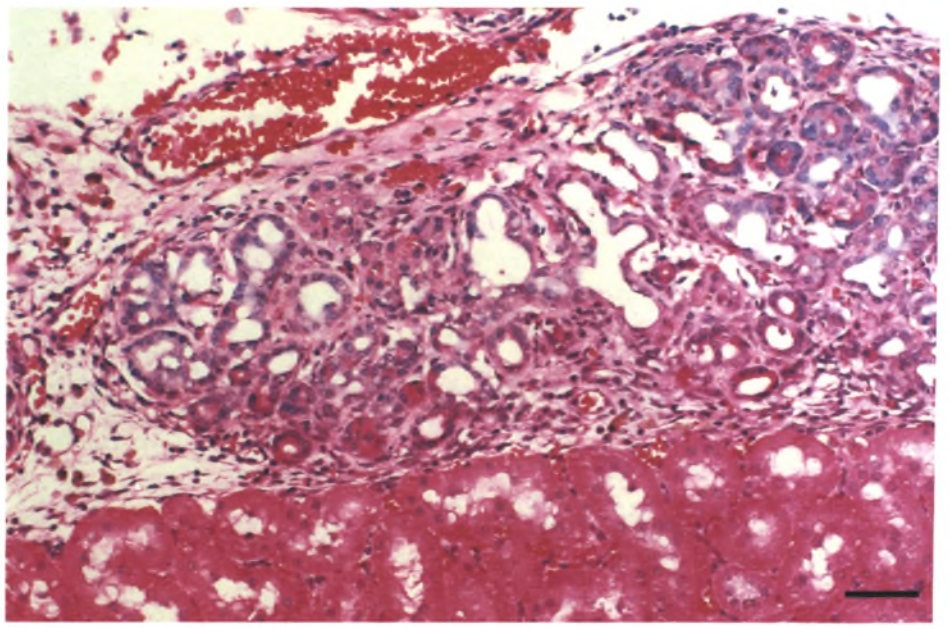

Figure 48 - Group 6: MMF and CsA therapy (50 mg/kg/d and 2 mg/kg/d) not only prevented graft rejection but resulted in an infiltrate free graft at 14 days post-transplantation. (H&E x100). Scale bar = 90 μm.

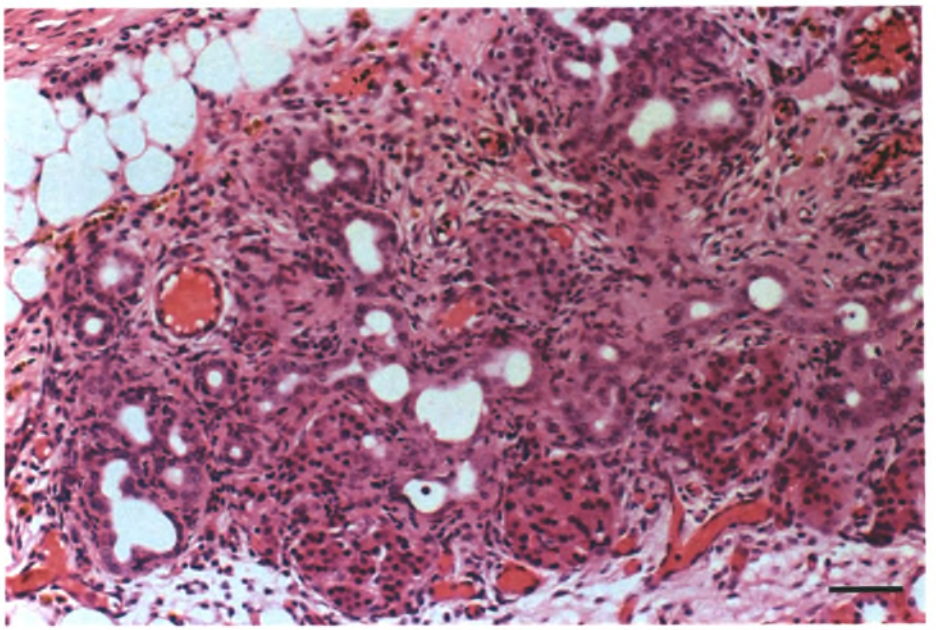

Figure 49 - Group 7: MMF and CsA therapy (50 mg/kg/d and 2 mg/kg/d) maintained an infiltrate free graft and allowed for excellent development of the islets of Langerhans 30 days post–transplantation. Perigraft fat formation is visible. (H&E x 100). Scale bar = 90 μm.

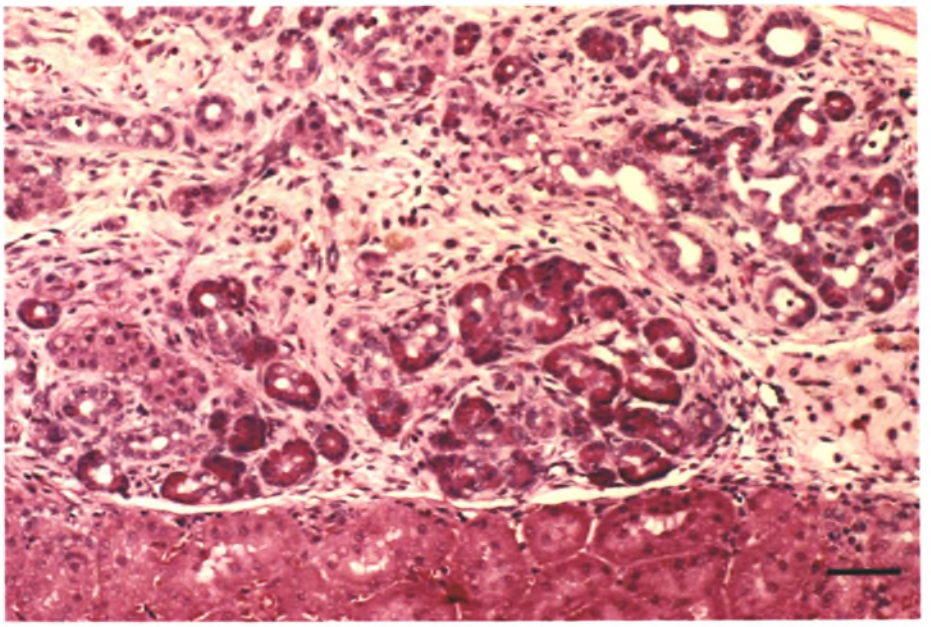

Figure 50 - Group 17: In the PVG rat, daily cyclosporine injections of 5 mg/kg/d effectively suppressed graft rejection resulting in minimal graft infiltration by lymphocytes (H&E x100). Scale bar = 90 μm.

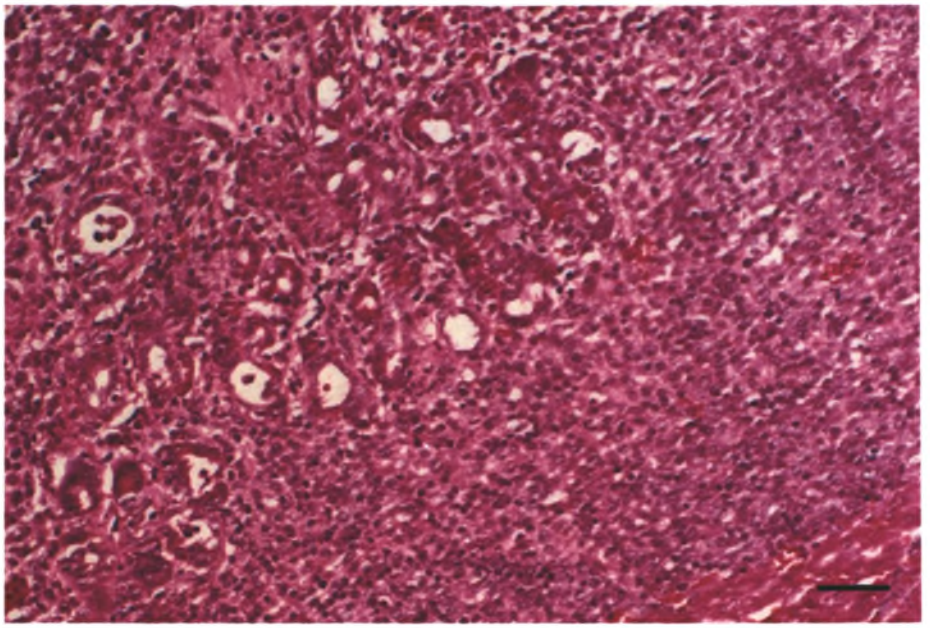

Figure 51: Group 18 - MMF monotherapy (oral 50 mg/kg/d) shows a heavy inter- and intra-graft mononuclear cell infiltrate similar to that seen in the SD rats (group 5). A few dilated ducts and islets were still present (H&E x100). Scale bar = 90 μm.

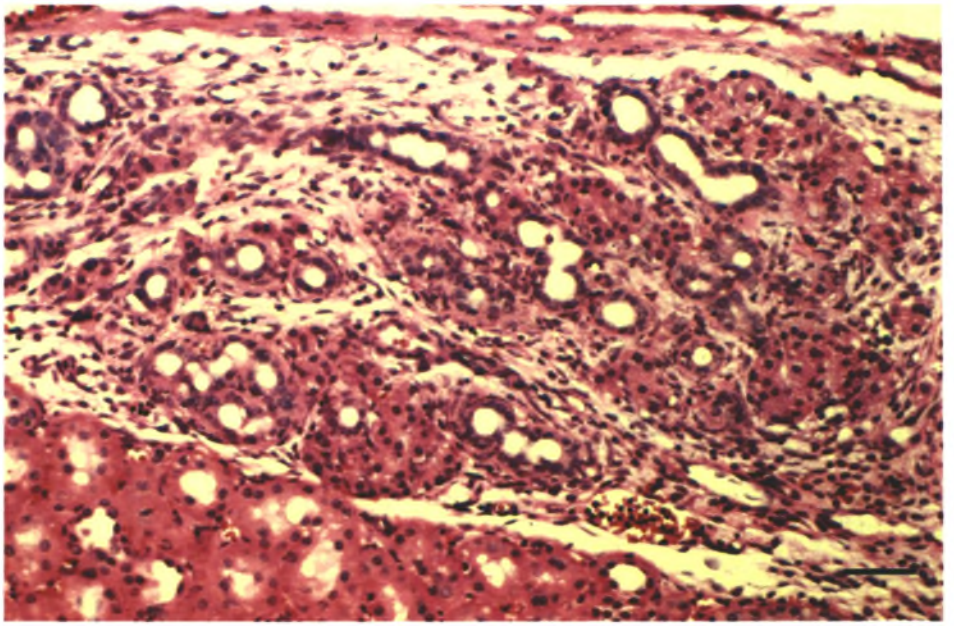

Figure 52: Group 19 - MMF and CsA therapy (50 mg/kg/d and 2 mg/kg/d) effectively suppressed graft rejection allowing for excellent development of islets within an infiltrate free graft while the exocrine tissue shows the typical signs of atrophy at 14 days post-transplantation (H&E x100). Scale bar = 90 μm.

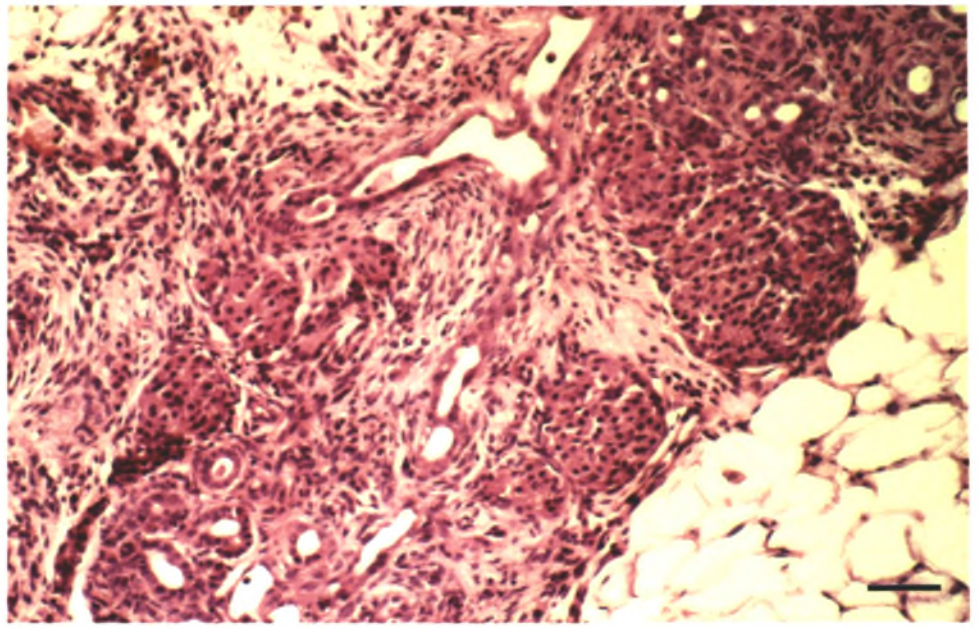

Figure 53: Group 20 - MMF and CsA therapy (50 mg/kg/d and 2 mg/kg/d) effectively prevents rejection at 30 days post-transplantation and allows for the development of islets of Langerhans, which are present within a fibrous stroma. The presence of perigraft fat, possibly due to ectopic insulin secretion, into the surrounding interstitium was a constant finding in this group (H&E x 100). Scale bar = 90 μm.

3.3. Islet viability and function was demonstrated in the combination therapy group (group 20) by electron microscopy Figure 54.

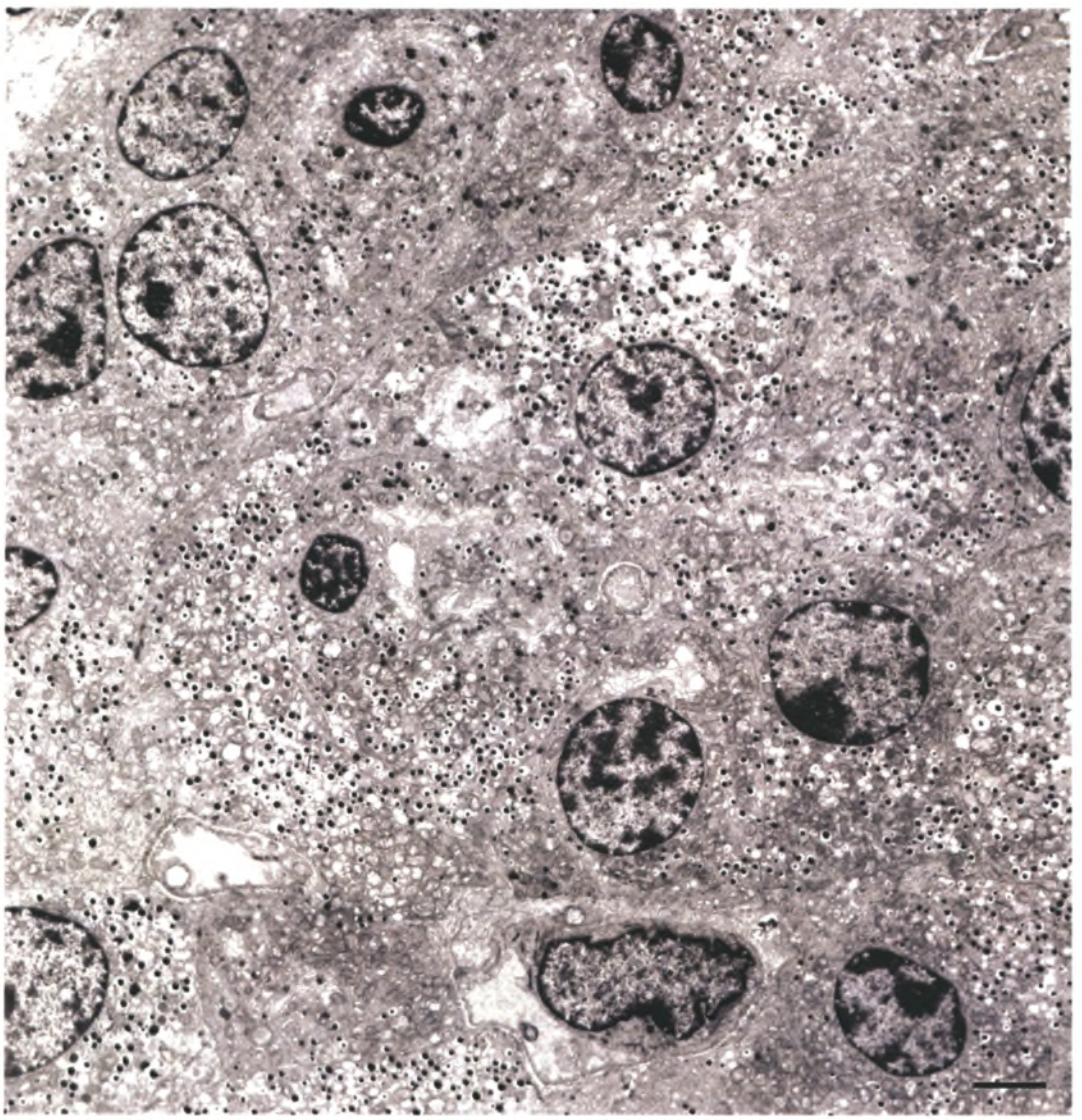

Figure 54: Group 20 - Electron microscopy of the islets of Langerhans following MMF and CsA therapy (50 mg/kg/d and 2 mg/kg/d) showed viable and well granulated endocrine cells. The majority of the endocrine cells contained typical dense core insulin-like granules. A capillary and endothelial cell demonstrates a well vascularised structure (x4500). Scale bar = 2.2 μm.

SECTION 4.

RESULTS OF THE ANTI-CD4 (W3/25) MONOCLONAL ANTIBODY IMMUNESUPPRESSION TRANSPLANTATION GROUPS.

W3/25 is a mouse anti -rat CD4 monoclonal antibobdy, which specifically binds to domain 1 of the CD4 molecule.

Flow cytometry of the peripheral blood lymphocytes demonstrated an 18% reduction of $CD2^{+}CD4^{+}$lymphocytes from the peripheral blood within 10 minutes (Figures 55d, 55e and 55f) of a single 500 μg intravenous injection with W3/25. Daily injection with W3/25 further reduced the $CD2^{+}CD4^{+}$lymphocytes from the peripheral blood (Figures 55c, 55f, 55i and 55l).

4.1. The pharmacokinetic effect of W3/25, as demonstrated by flow cytometry, is reflected in tables 14, 15 and 16.

Whole Blood Lymphocyte Flow Cytometry W3/25 Monotherapy Groups

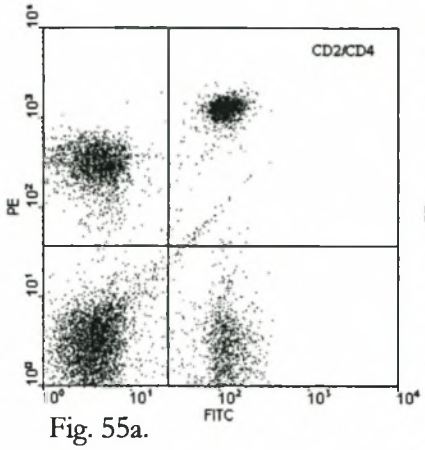

Fig. 55a.

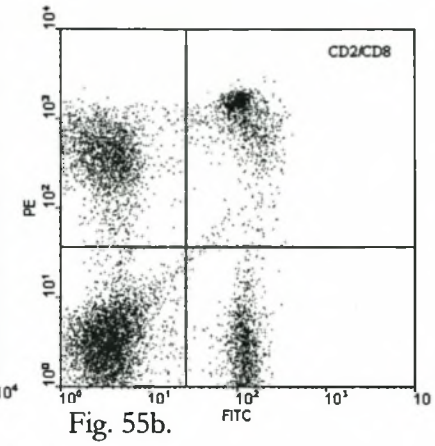

Fig. 55b.

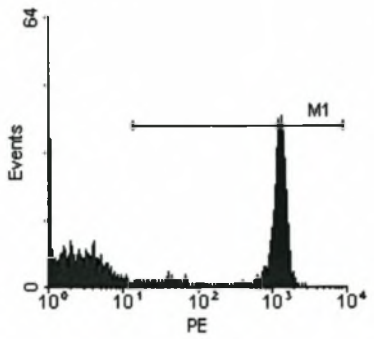

Fig. 55c.

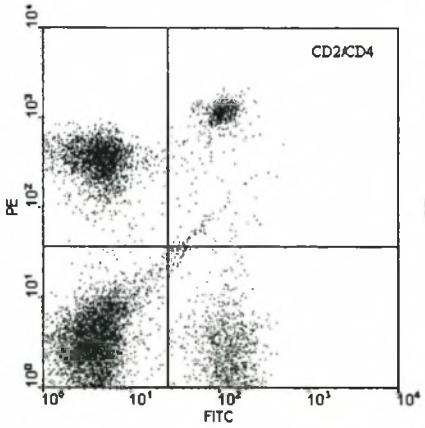

Fig. 55d.

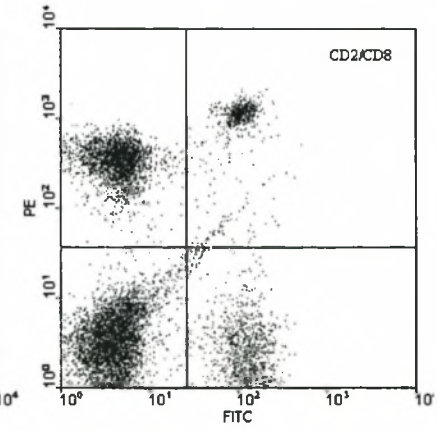

Fig. 55e.

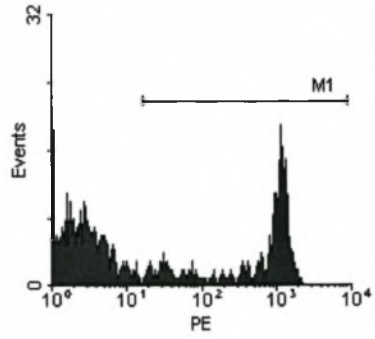

Fig. 55f.

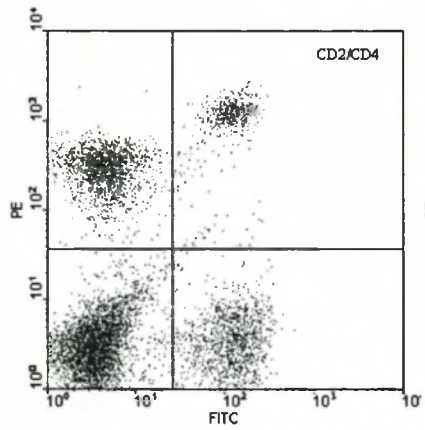

Fig. 55g.

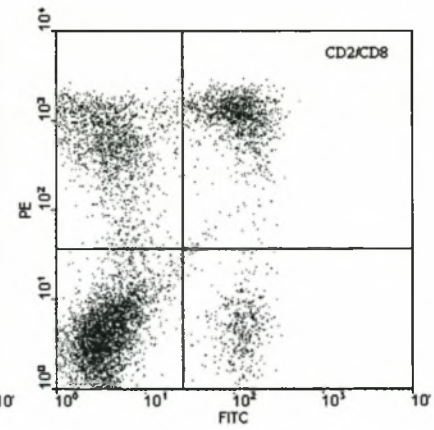

Fig. 55h.

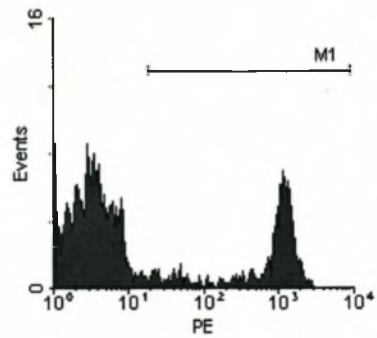

Fig. 55i.

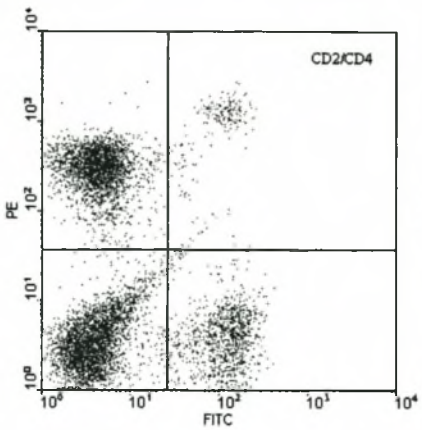

Fig. 55j.

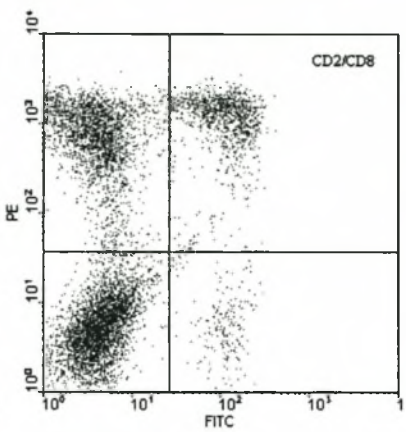

Fig. 55k.

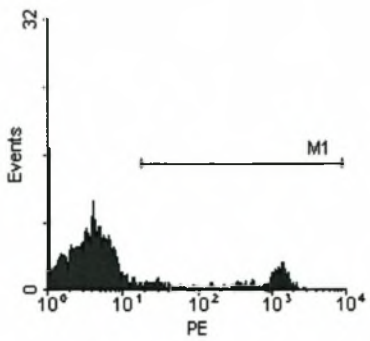

Fig. 55l.

Figure 55: W3/25 has a dramatic effect on the CD4 expression of $CD2^+CD4^+$ PBL's. The above dotplots and histograms (demonstrating the gated $CD2^+CD4^+$ PBL population) show the effect of 500µg W3/25 injections on a 450 g adult male SD rat. Figures 55a, 55b and 55c show the $CD2^+CD4^+$ and $CD2^+CD8^+$ PBL expression prior to W3/25 administration, which is 54.2% and 47.8% respectively. 10 minutes after a 500 µg intravenous injection of W3/25 (Figs 55d, 55e and 55f) the $CD2^+CD4^+$ PBL's have already declined to 36.2% while the $CD2^+CD8^+$ PBL's percentage has increased to 66%. 1 day after the commencement of W3/25 therapy the $CD2^+CD4^+$ PBL's continue to decline further to 28.7% and conversely the $CD2^+CD8^+$ PBL's percentage continues to increase 70.1%. Daily injections for 30 days result in severe $CD2^+CD4^+$ lymphocytopenia as demonstrated in figures 55j and 55l (16.1% $CD2^+CD4^+$ PBL's) and an marked increase of $CD2^+CD8^+$ to 84.5%

The $CD2^+CD4^+$ decrease, and the $CD2^+CD8^+$ PBL percentage increase, correlated significantly SD (groups 6 and 7) - non-parametric Spearman Rank test (correlation $r = -0.8655$ and the two-tailed p value for r was < 0.0001) and ($r = -0.9652$ and a two-tailed p value for r was < 0.0001) in the PVG (groups 19 and 20).

Post-Tx	CD2	CD2CD4	CD2CD8	CD4/CD8	CD4/CD25
Day 0	35.3 ± 1.7	53.8 ± 1.0	46.1 ± 1.0	1.2 ± 0.01	6.4 ± 0.9
Day 1	32.5 ± 2.2 (p=0.3)	41.8 ± 4.1 (p=0.008)	58.4 ± 3.8 (p=0.008)	0.7 ± 0.1 (p=0.008)	7.2 ± 1.0 (p=0.2)
Day 3	28.1 ± 1.8 (p=0.2)	34.8 ± 2.3 (p=0.008)	60.3 ± 2.3 (p=0.008)	0.6 ± 0.1 (p=0.008)	7.9 ± 1.0 (p=0.1)
Day 7	29.2 ± 3.2 (p=0.3)	33.1 ± 2.3 (p=0.008)	67.8 ± 2.2 (p=0.008)	0.5 ± 0.1 (p=0.008)	7.0 ± 1.3 (p=0.1)
Day 14	27.8 ± 2.0 (p=0.1)	30.9 ± 2.2 (p=0.008)	68.3 ± 2.8 (p=0.008)	0.5 + 0.1 (p=0.008)	7.6 ± 1.0 (p=0.1)

Table 14: Group 8 - Two colour flow cytometric PBL analysis of W3/25 monotherapy 200 µg/day (DA ⇒SD).

Post-Tx	CD2	CD2CD4	CD2CD8	CD4/CD8	CD4/CD25
Day 0	34.7 ± 2.5	50.4 ± 2.0	50.8± 2.3	1.0 ± 0.1	5.1 ± 0.7
Day 1	25.2 ± 0.9 (p=0.2)	25.5± 3.2 (p=0.008)	67.9 ± 3.1 (p=0.008)	0.4 ± 0.1 (p=0.008)	9.4 ± 1.5 (p=0.03)
Day 3	25.6 ± 1.6 (p=0.2)	19.8 ± 0.9 (p=0.008)	75.6 ± 1.3 (p=0.008)	0.3 ± 0.1 (p=0.008)	8.6 ± 0.6 (p=0.02)
Day 7	23.3 ± 1.0 (p=0.09)	17.8 ± 0.9 (p=0.008)	78.9 ± 1.2 (p=0.008)	0.2 ± 0.1 (p=0.008)	7.8 ± 0.8 (p=0.03)
Day 14	24.4 ± 3.3 (p=0.2)	17.3 ± 1.9 (p=0.008)	87.7 ± 3.7 (p=0.008)	0.2 + 0.01 (p=0.008)	7.8 ± 0.8 (p=0.03)
Day 21	21.4 ± 1.0 (p=0.03)	16.5 ± 1.7 (p=0.008)	81.9 ± 1.0 (p=0.008)	0.2 ± 0.01 (p=0.008)	8.2 ± 0.6 (p=0.03)
Day 30	20.9 ± 1.3 (p=0.03)	16.8 ± 1.2 (p=0.008)	82.5 ± 0.7 (p=0.008)	0.2 ± 0.001 (p=0.008)	9.0 ± 1.0 (p=0.02)

Table 15: Groups 9 and 10 - Two colour flow cytometric PBL analysis of W3/25 monotherapy 500 µg/day (DA ⇒SD).

Post-Tx	CD2	CD2CD4	CD2CD8	CD4/CD8	CD4/CD25
Day 0	40.9 ± 1.3	72.3 ± 1.0	21.1 ± 1.0	3.5 ± 0.2	6.4 ± 0.3
Day 1	27.8 ± 1.1 (p=0.008)	60.8 ± 1.9 (p=0.03)	36.6 ± 2.2 (p=0.008)	1.7 ± 0.2 (p=0.008)	6.2 ± 0.4 (p=0.9)
Day 3	25.7 ± 1.2 (p=0.008)	56.9 ± 1.6 (p=0.008)	36.1 ± 1.4 (p=0.008)	1.6 ± 0.1 (p=0.008)	6.7 ± 0.3 (p=0.4)
Day 7	21.8 ± 2.0 (p=0.008)	50.0 ± 1.9 (p=0.008)	38.8 ± 1.3 (p=0.008)	1.3 ± 0.1 (p=0.008)	7.0 ± 0.7 (p=0.4)
Day 14	18.3 ± 1.1 (p=0.008)	42.9 ± 1.6 (p=0.008)	43.4 ± 0.7 (p=0.008)	1.0 ± 0.05 (p=0.008)	8.4 ± 1.0 (p=0.2)
Day 21	15.3 ± 1.7 (p=0.008)	34.5 ± 4.3 (p=0.008)	57.8 ± 4.5 (p=0.008)	0.6 ± 0.1 (p=0.008)	7.9 ± 0.8 (p=0.2)
Day 30	15.7 ± 0.9 (p=0.008)	28.7 ± 3.8 (p=0.008)	64.7 ± 3.9 (p=0.008)	0.5 ± 0.1 (p=0.008)	7.8 ± 1.3 (p=0.7)

Table 16: Groups 21 and 22 - Two colour flow cytometric PBL analysis of W3/25 monotherapy 500 μg/day (DA ⇒ PVG).

4.2. The efficacy of W3/25 as an immunosuppressant in preventing graft rejection in both rat foetal rat pancreatic models is demonstrated by the histology of the harvested grafts in figures 56 to 60.

Figure 56: Group 8 - A photomicrograph demonstrating the presence of renal subcapsular grafts following daily W3/25 (200 µg/d) injections, at 14 days post-transplantation. Note the clear glandular appearance of the grafts under the kidney capsule. Scale 1:3.5

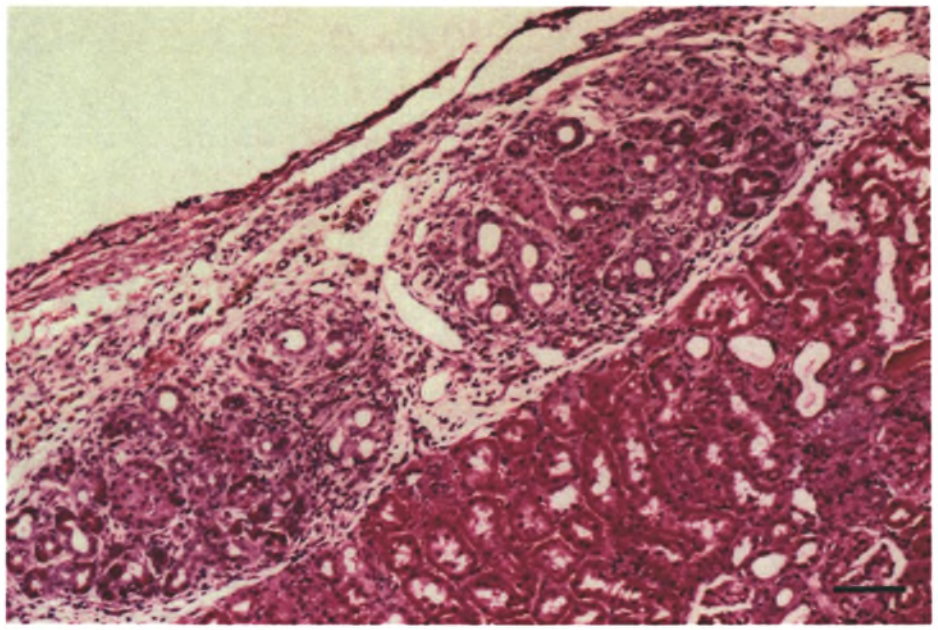

Figure 57: Group 8: Following daily W3/25 (200 µg/d) injections,at 14 days post-transplantation, histology of the graft showed well preserved islets. The exocrine tissue showed ductal dilatation and some destruction. A light to moderate inter- and intragraft mononuclear cell infiltrate is present. This may be indicative of early rejection but may well reflect resolving inflammatory cells following transplantation (H&E x50). Scale bar = 170 µm.

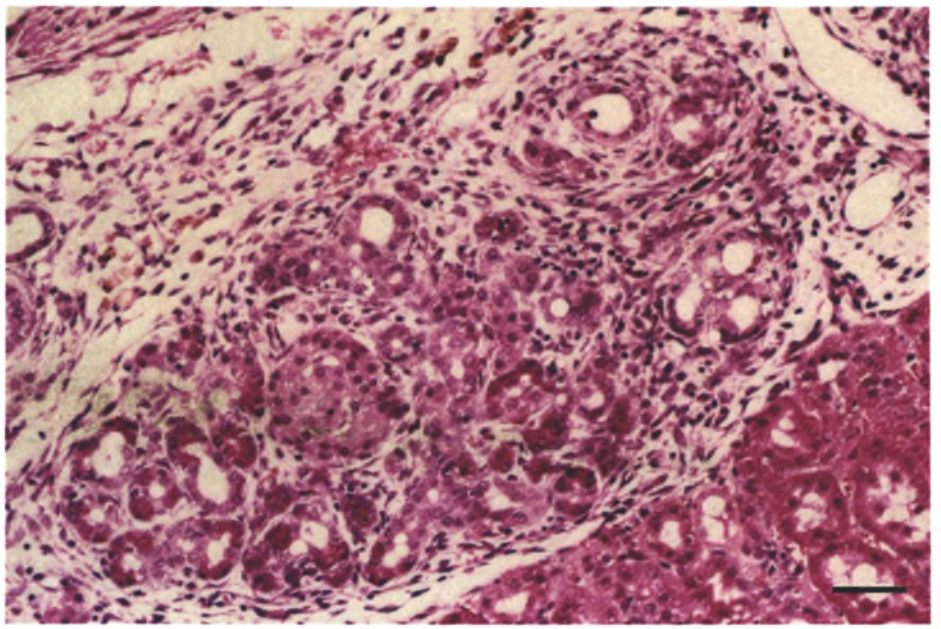

Figure 58: Group 8 - Higher magnification of the graft clearly illustrates the presence of the islets and the mononuclear cell infiltrate present within the graft. Exocrine tissue undergoing artophy is still present within the graft (H&E x100). Scale bar = 90 μm.

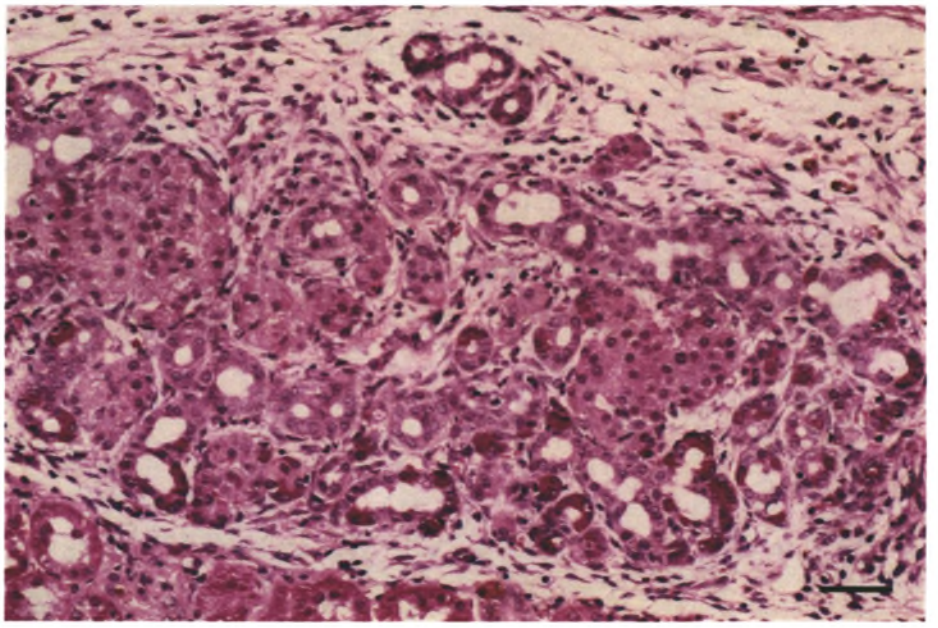

Figure 59: Group 9 - Demonstrated excellent graft survival following daily 500 μg/d W3/25 injections. At 14 days post-transplantation, the grafts showed the presence of well developed islets and atrophying exocrine tissue. A light peri- and intragraft infiltrate is present (H&E x100). Scale bar = 90 μm.

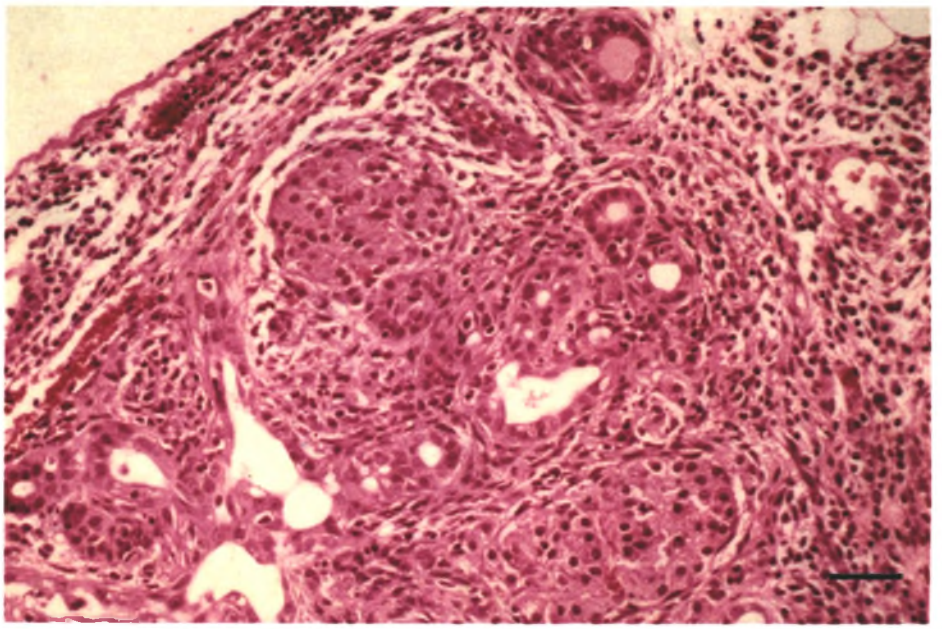

Figure 60: Group 10 - Histology of the grafts at 30 days following daily 500 µg/d W3/25 injections showed that the grafts consisted mostly of well developed islets. The exocrine tissue has atrophied and is absent except for a few ducts still present. A moderate peri- and intragraft infiltrate is present (H&E x100). Scale bar = 90 µm.

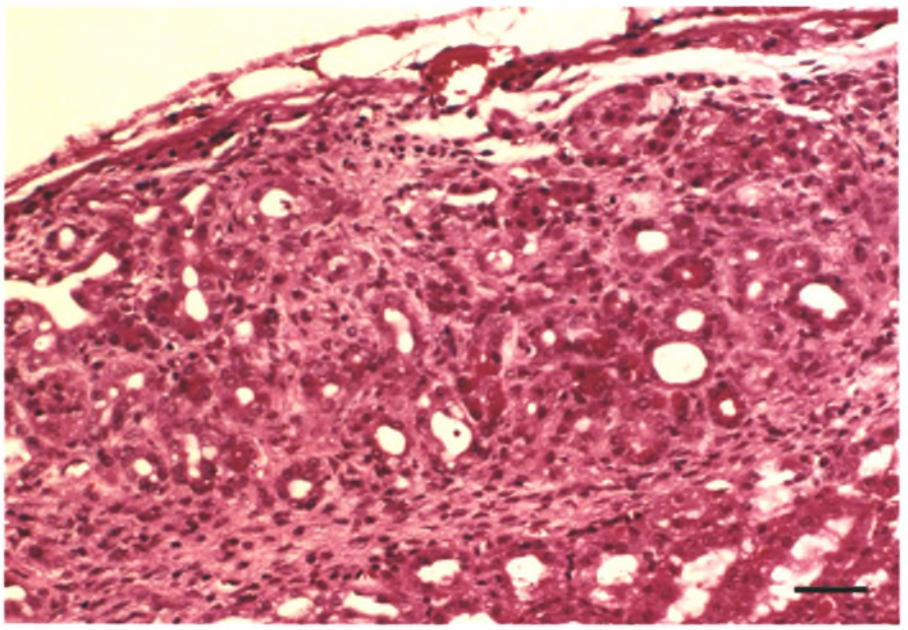

Figure 61: Group 21 - Graft survival at 14 days post-transplantation, following daily 500 µg W3/25 injections, showed similar graft survival as seen in group 6 (SD rats). A mild peri- and intragraft infiltrate is present (H&E x100). Scale bar = 90 µm.

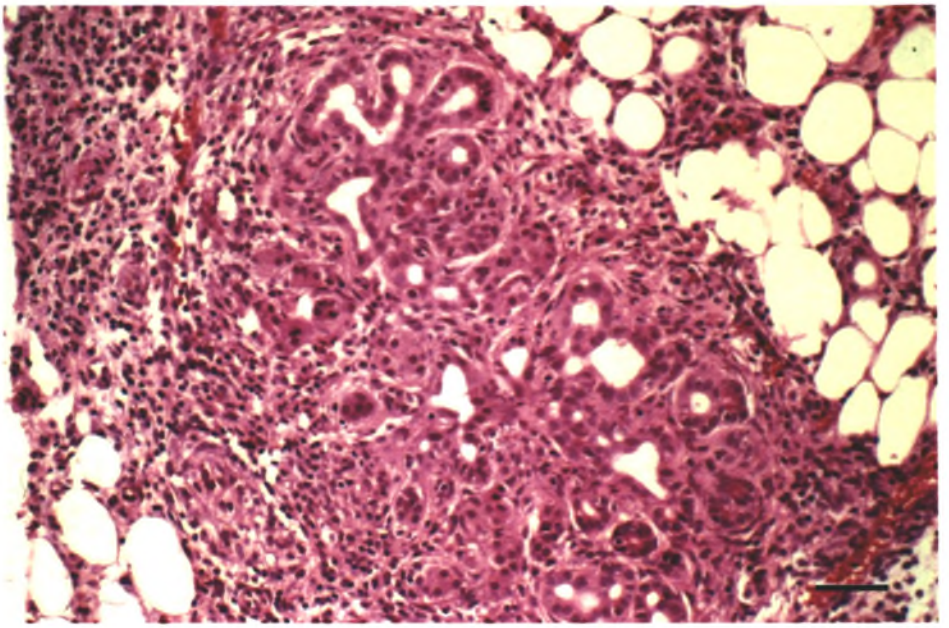

Figure 62: Group 22 - Good graft survival at 30 days post-transplantation, following daily 500 µg W3/25 injections, showed a moderate lymphocytic graft infiltrate [DA ⇒ PVG]. Perigraft WAT is present (H&E x100). Scale bar = 90 µm.

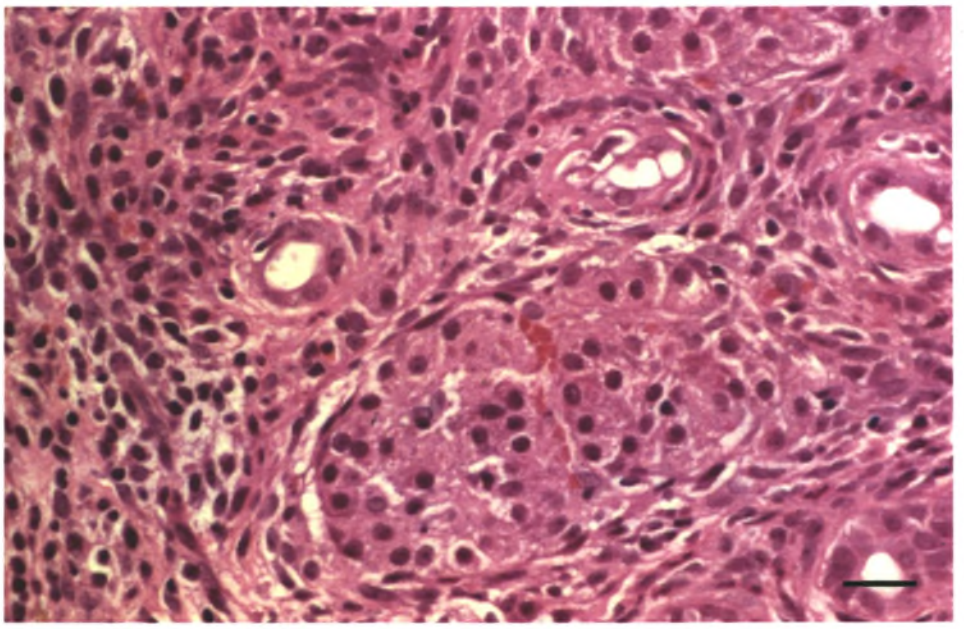

Figure 63: Group 22 - Higher magnification shows the presence of well developed islets of Langerhans within a fibrous stroma 30 days post-transplantation (H&E x200). Scale bar = 20 µm.

SECTION 5.

RESULTS OF DONOR SPECIFIC TRANSFUSION (DST), CYCLOSPORINE (CSA) AND ANTI-CD4 (W3/25) INDUCTION THERAPY GROUPS – DONOR SPECIFIC ANTIGEN INDUCED UNRESPONSIVENESS.

In these groups, by combining CsA with its broader immunosuppressive effect, and anti-CD4, known to act synergistically with CsA with DST, donor specific tolerance was demonstrated. To test the efficacy of this treatment regime, a single 3ml DST transfusion was given 1 day pre-transplantation. In the combination groups, DST was followed by 5 days of either CsA (5mg/kg/d) or W3/25 (500 μg/d) or a combination of the two. Thereafter no further immunesuppression was given until harvesting at either 14 or 30 days post-transplantation.

5.1. Flow cytometry:

DST had no measurable effect on the peripheral blood T-Cell immunophenotypes (pre-DST vs. DST 14-days post-DST; p=NS) as reflected in tables 17 and 18.

In comparison to the pre-treatment levels, DST and W3/25 induction therapy resulted in a significant decline of $CD2^+CD4^+$ PBL cell percentage at 14-days post-transplantation from 53.0% ± 0.7 to 28.5% ± 1.7; p=0.03 (group 13 – Table 21) and 72.3% ± 2.3 to 65.4% ± 1.4; p=0.05 (group 25 – Table 22).

The pharmacokinetic effect of W3/25 therapy and DST on the peripheral blood $CD2^+CD4^+$ T-Cells (Tables 21 and 22) was significantly enhanced by the addition of CsA and DST in the SD groups (14 and 15 – Table 23) declining from 52.7% ± 2.9 to 14.4% ± 2.5 (p=0.003) while in the

PVG groups (26 and 27 – Table 24) the PBL $CD2^{+}CD4^{+}$ cells had recovered from 73.2 ± 2.0 to 67.2 ± 1.3 (p=0.1).

Whole Blood Lymphocyte Flow Cytometry DST and Combination Groups

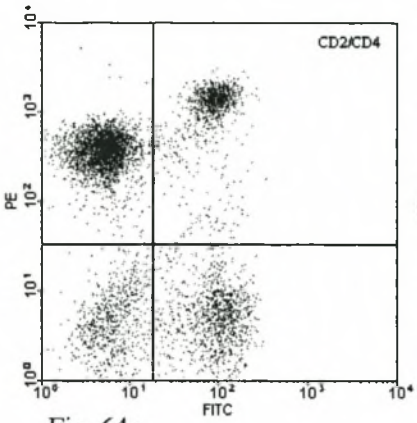

Fig. 64a.

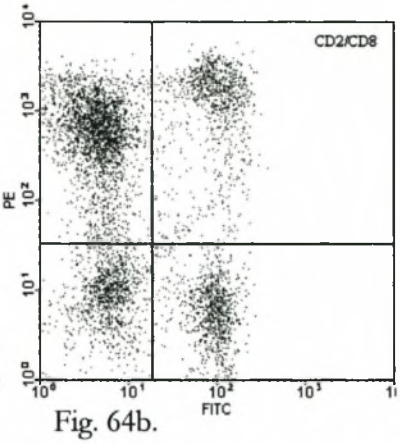

Fig. 64b.

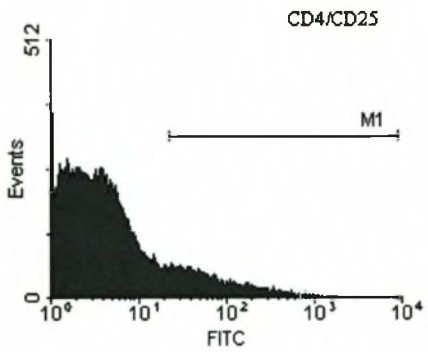

Fig. 64c.

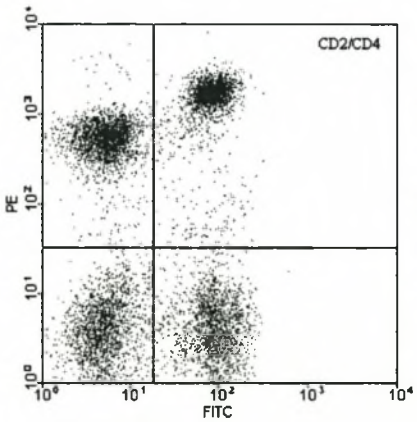

Fig. 64d.

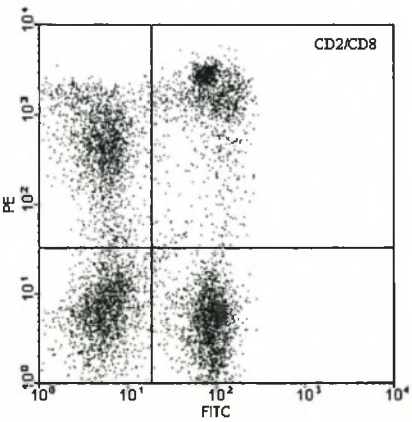

Fig. 64e.

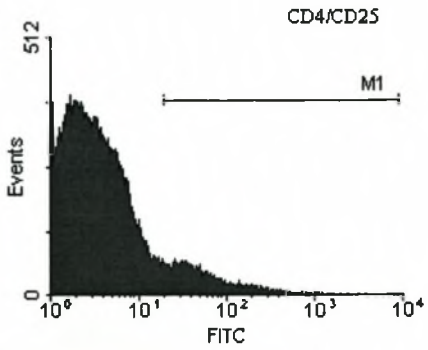

Fig. 64f.

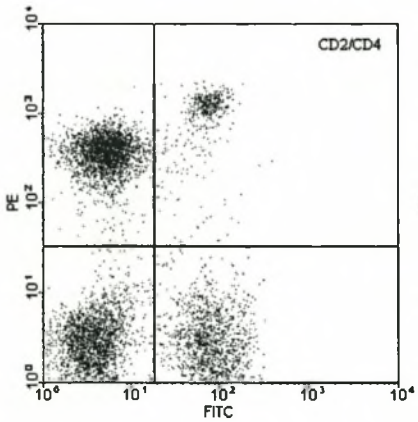

Fig. 64g.

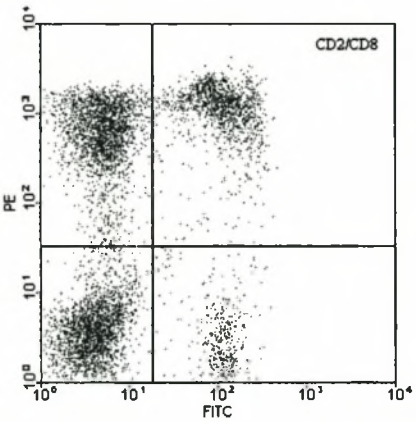

Fig. 64h.

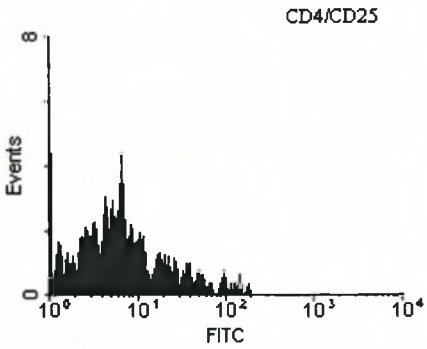

Fig. 64i.

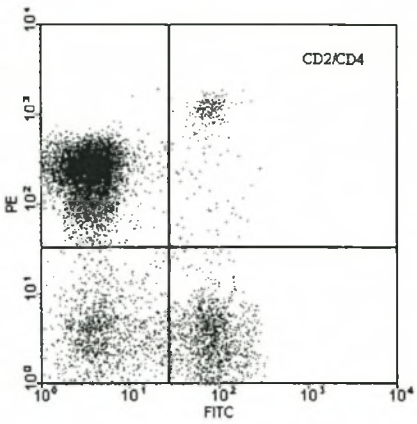

Fig. 64j.

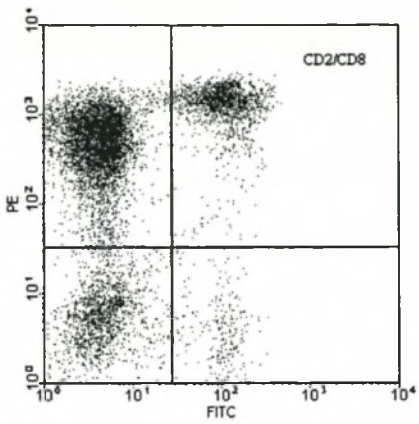

Fig. 64k.

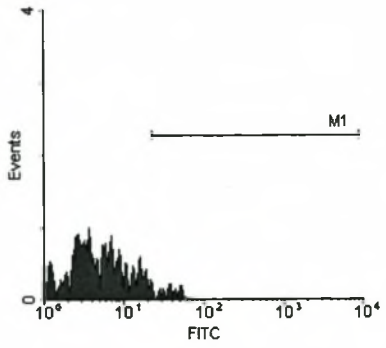

Fig. 64l.

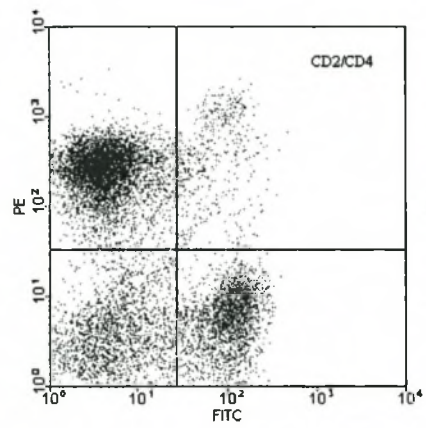

Fig. 64m.

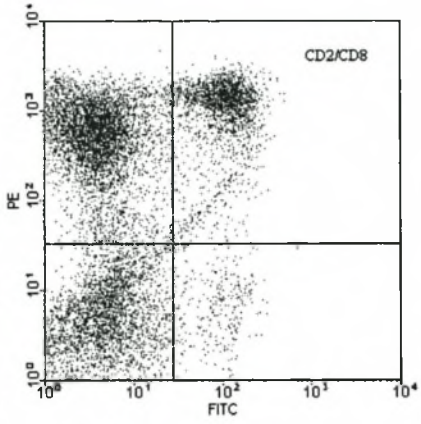

Fig. 64n.

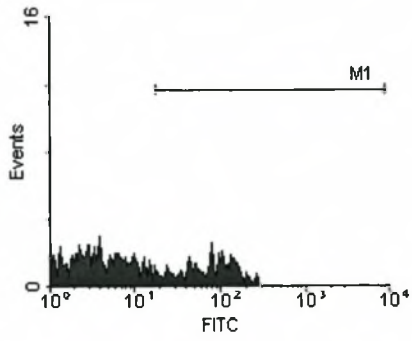

Fig. 64o.

Figure 64: The above flow cytometric dotplots and histograms demonstrate the effect of DST as a monotherapy or in combination with either CsA or W3/25 induction or as a triple combination induction therapy protocol on the SD rat (Groups 11, 12, 13, 14 and 15). A single donor specific transfusion (Fig 64a, 64b and 64c) had no significant effect on the PBL immunophenotype percentages. DST in combination with CsA resulted in a significant decline in $CD2^+CD4^+$ (52.0% ± 1.3 to 21.7 ± 1.4; p=0.05) and the CD4/CD8 ratio (1.1 ± 0.1 to 0.3 ± 0.1; p=0.05) 1 day post-transplantation, (group 12 - SD group) but had recovered by day 7 (Figures 64d, 64e and 64f). In the PVG rats (group 25) no significant effect was demonstrated. A marked decline in the $CD2^+CD4^+$ PBL's (53.0% ± 0.7 to 28.5% ± 1.7; p=0.03) is clearly noticeable (fig 64g: left upper quadrant) following DST and W3/25 induction therapy (group 13) 14 days post-transplantation. Conversely the $CD2^+CD8^+$ lymphocytes had increased from 47.3% ± 0.1 to 69.9 ± 2.1; p=0.03. In the DST, CsA and W3/25 induction therapy group (groups 14 and 15) the loss of $CD2^+CD4^+$ cells from the PBL population was significant both at 14 and 30 days post-

was significant both at 14 and 30 days post-transplantation (Figs 64j and 64m). This resulted in a significant increase of both the $CD2^+CD8^+$ lymphocytes (figs 64k and 64n). Of interest is the increase, although not significant, in the percentage of $CD4^+CD25^+$ PBL's (figure 64o) compared to the pre-transplantation values 30 days post-transplantation (from 5.8% ± 0.5 to 12.3% ± 1.8; p=0.2).

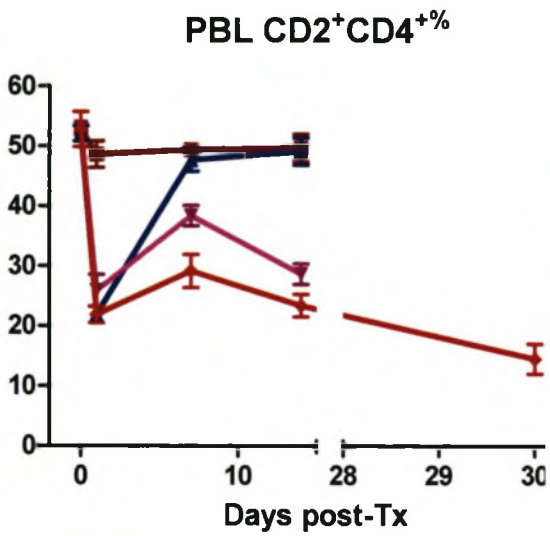

Fig. 65a.

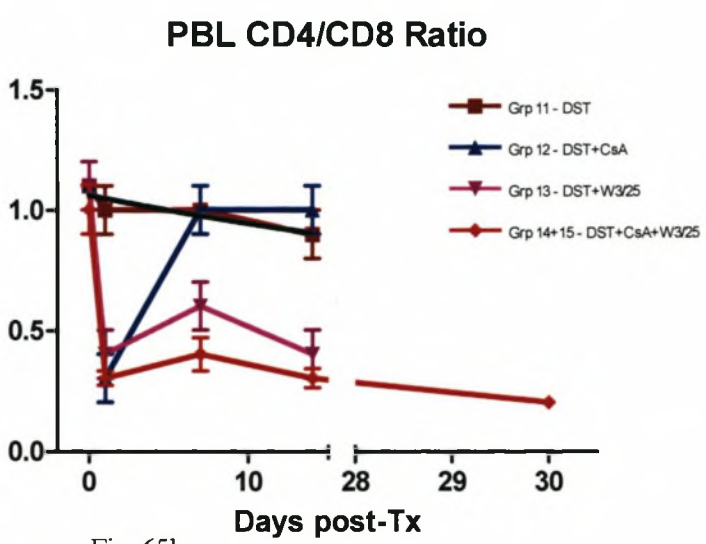

Fig. 65b.

Figure 65: Graphs comparing the pharmacokinetic effect of DST (group 11), DST+ CsA (group 12), DST+W3/25 (group 13) and DST+CsA+W3/25 (group 14) therapy on the PBL $CD2^+CD4^+$ percentage (figure 65a) and the CD4/CD8 ratio (figure 65b) of the SD rat groups.

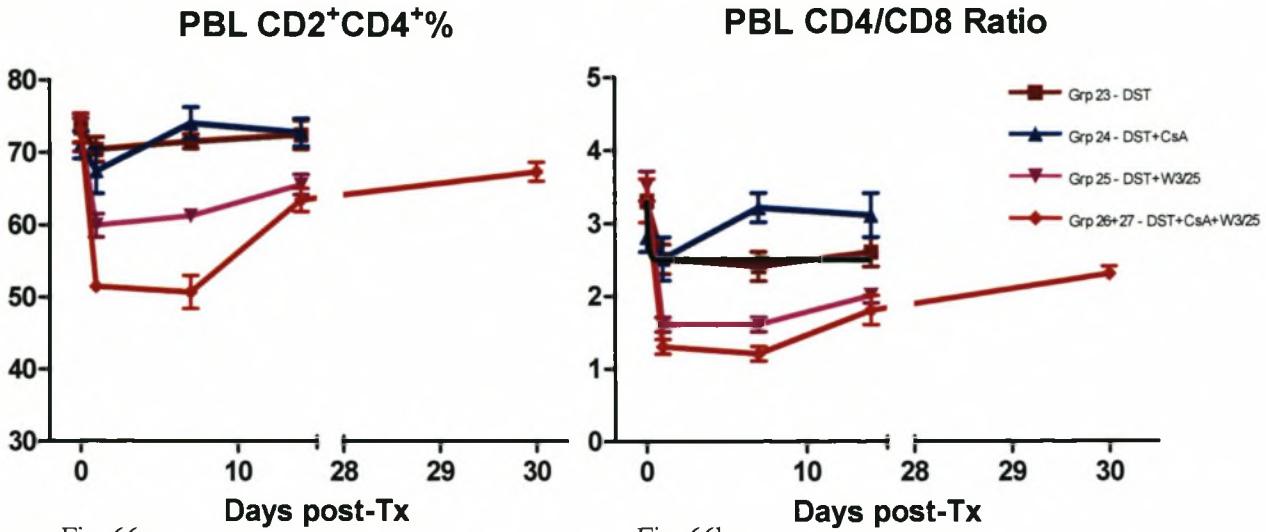

Fig. 66a. Fig. 66b.

Figure 66: Graph comparing the pharmacokinetic effect of DST (group 23), DST+ CsA (group 24), DST+W3/25 (group 25) and DST+CsA+W3/25 (group 26) therapy on the PBL $CD2^+CD4^+$ percentage (figure 66a) and CD4/CD8 ratio of the PVG rat groups (figure 66b).

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	37.3 ± 0.6	52.7 ± 0.9	47.4 ± 0.4	1.1 ± 0.1	6.0 ±0.2
Day 1	35.0 ± 2.6 (p=0.7)	48.5 ± 2.2 (p=0.3)	51.4 ± 1.8 (p=0.3)	1.0 ± 0.1 (p=0.3)	7.3 ± 0.8 (p=0.3)
Day 7	38.3 ± 3.7 (p=0.9)	49.3 ± 0.7 (p=0.4)	49.6 ± 0.9 (p=0.5)	1.0 ± 0.1 (p=0.3)	7.1 ± 0.5 (p=0.4)
Day 14	33.7 ± 1.8 (p=0.5)	49.6 ± 2.3 (p=0.4)	52.4 ± 2.2 (p=0.2)	0.9 ± 0.1 (p=0.2)	7.1 ± 0.3 (p=0.4)

Table 17: Group 11 - Two colour flow cytometric PBL analysis following DST (DA ⇒ SD) monotherapy. Values are mean ± SEM.

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	39.5 ± 2.6	73.3 ± 2.0	22.6 ±1.8	3.3 ± 0.3	6.0 ±0.2
Day 1	37.4 ± 2.4 (p=0.8)	70.3 ± 1.7 (p=0.3)	28.2 ± 1.7 (p=0.06)	2.5 ± 0.2 (p=0.06)	5.7 ± 0.1 (p=0.3)
Day 7	39.0 ± 1.7 (p=0.8)	71.4 ± 1.0 (p=0.2)	29.9 ± 1.9 (p=0.05)	2.4 ± 0.2 (p=0.06)	5.8 ± 0.2 (p=0.6)
Day 14	39.4 ± 2.6 (p=0.8)	72.3 ± 2.0 (p=0.9)	28.5 ± 1.7 (p=0.06)	2.6 ± 0.2 (p=0.08)	5.4 ± 0.2 (p=0.1)

Table 18: Group 23 - Two colour flow cytometric PBL analysis following DST (DA ⇒ PVG) monotherapy. Values are mean ± SEM.

Day Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	38.3 ± 0.7	52.0 ± 1.3	47.4 ± 0.7	1.1 ± 0.1	5.7 ± 0.3
Day 1	27.5 ± 1.8 (p=0.08)	21.7 ± 1.4 (p= 0.05)	75.9 ± 2.0 (p=0.06)	0.3 ± 0.1 (p=0.05)	9.2 ± 1.6 (p=0.09)
Day 7	43.0 ± 2.1 (p=0.1)	47.6 ± 1.9 (p=0.6)	49.0 ± 2.2 (p=0.6)	1.0 ± 0.1 (p=0.5)	6.9 ± 0.6 (p=0.3)
Day 14	40.9 ± 0.9 (p=0.3)	49.0 ± 2.3 (p=0.2)	52.4 ± 2.0 (p=0.2)	1.0 ± 0.1 (p=0.1)	6.0 ± 0.8 (p=0.9)

Table 19: Group 12 - Two colour flow cytometric PBL analysis following DST + CsA induction therapy for 5 days (DA ⇒ SD). Values are mean ± SEM.

Day Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	43.6 ± 3.6	70.9 ± 1.9	25.8 ± 2.1	2.8 ± 0.2	6.7 ± 0.2
Day 1	28.7 ± 3.4 (p=0.1)	67.3 ± 3.1 (p=0.1)	26.9 ± 3.0 (p=0.1)	2.5 ± 0.3 (p=0.2)	7.3 ± 1.3 (p=0.2)
Day 7	32.1 ± 2.4 (p=0.1)	73.9 ± 2.2 (p=0.4)	23.3 ± 0.9 (p=0.3)	3.2 ± 0.2 (p=0.3)	7.1 ± 0.6 (p=0.6)
Day 14	37.6 ± 1.3 (p=0.2)	72.6 ± 1.9 (p=0.5)	23.9 ± 2.1 (p=0.4)	3.1 ± 0.3 (p=0.4)	8.3 ± 1.1 (p=0.6)

Table 20: Group 24 - Two colour flow cytometric PBL analysis following DST + CsA induction therapy for 5 days (DA ⇒ PVG). Values are mean ± SEM.

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	37.3 ± 1.8	53.0 ± 0.7	47.3 ± 0.4	1.1 ± 0.1	6.6 ± 0.3
Day 1	26.8 ± 2.4 (p=0.03)	25.6 ± 2.7 (p=0.02)	74.7 ± 2.7 (p=0.02)	0.4 ± 0.1 (p=0.02)	5.8 ± 0.1 (p=0.1)
Day 7	48.6 ± 2.7 (p=0.1)	38.2 ± 1.7 (p=0.06)	60.7 ± 0.6 (p=0.06)	0.6 ± 0.1 (p=0.03)	5.9 ± 0.5 (p=0.2)
Day 14	26.6 ± 1.0 (p=0.03)	28.5 ± 1.7 (p=0.03)	69.6 ± 1.4 (p=0.03)	0.4 ± 0.1 (p=0.03)	9.6 ± 0.9 (p=0.06)

Table 21: Group 13 - Two colour flow cytometric PBL analysis following DST + W3/25 µg/day combination therapy for 5 days (DA ⇒ SD). Values are mean ± SEM.

Post –Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	40.9 ± 4.0	72.3 ± 2.3	21.1 ± 1.0	3.5 ± 0.2	6.4 ± 0.3
Day 1	27.8 ± 1.0 (p=0.05)	59.8 ± 1.6 (p=0.03)	38.1 ± 1.7 (p=0.03)	1.6 ± 0.1 (p=0.02)	6.2 ± 0.4 (p=0.7)
Day 7	30.9 ± 0.7 (p=0.07)	61.1 ± 0.7 (p=0.03)	37.8 ± 1.2 (p=0.02)	1.6 ± 0.1 (p=0.02)	7.2 ± 0.4 (p=0.2)
Day 14	34.7 ± 1.3 (p=0.1)	65.4 ± 1.4 (p=0.05)	33.4 ± 1.5 (p=0.05)	2.0 ± 0.1 (p=0.05)	6.6 ± 0.6 (p=0.9)

Table 22: Group 25 - Two colour flow cytometric PBL analysis following DST + W3/25 µg/day combination therapy for 5 days (DA ⇒ PVG). Values are mean ± SEM.

Post Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	41.1 ± 2.8	52.7 ± 2.9	52.5 ± 1.3	1.0 ± 0.1	5.8 ± 0.5
Day 1	27.5 ± 1.8 (p=0.008)	21.7 ± 1.4 (p=0.003)	75.9 ± 2.0 (p=0.003)	0.3 ± 0.03 (p=0.003)	9.2 ± 1.6 (p=0.2)
Day 7	17.8 ± 1.1 (p=0.03)	29.0 ± 2.8 (p=0.008)	66.7 ± 3.4 (p=0.004)	0.4 ± 0.07 (p=0.008)	6.2 ± 0.8 (p=0.4)
Day 14	25.2 ± 3.3 (p=0.03)	23.2 ± 1.9 (p=0.008)	78.0 ± 1.9 (0.004)	0.3 ± 0.04 (p=0.008)	7.0 ± 0.7 (p=0.5)
Day 30	22.9 ± 1.6 (p=0.03)	14.4 ± 2.5 (p=0.003)	74.4 ± 3.0 (p=0.004)	0.2 ± 0.02 (p=0.003)	12.3 ± 1.8 (p=0.2)

Table 23: Groups 14 and 15 - Two-colour flow cytometric PBL analysis following DST + CsA + W3/25 induction therapy for 5 days (DA ⇒ SD). Values are mean ± SEM.

Day Tx	CD2	CD2/CD4	CD2/CD8	CD4/CD8	CD4/CD25
Day 0	39.5 ± 2.6	73.2 ± 2.0	22.6 ± 1.8	3.3 ± 0.3	7.5 ± 1.2
Day 1	22.3 ± 0.6 (p=0.008)	51.4 ± 0.9 (p=0.008)	39.4 ± 2.0 (p=0.008)	1.3 ± 0.1 (p=0.008)	6.3 ± 0.2 (p=0.5)
Day 7	18.6 ± 0.9 (p=0.008)	50.6 ± 2.3 (p=0.008)	44.2 ± 1.5 (p=0.008)	1.2 ± 0.1 (p=0.008)	7.9 ± 0.6 (p=0.3)
Day 14	22.2 ± 1.0 (p=0.008)	63.3 ± 1.6 (p=0.3)	35.0 ± 2.1 (p=0.02)	1.8 ± 0.2 (p=0.02)	7.9 ± 1.1 (p=0.9)
Day 30	25.0 ± 1.2 (p=0.008)	67.2 ± 1.3 (p=0.1)	28.9 ± 1.9 (p=0.2)	2.3 (0.4 (p=0.08)	6.7 (0.4 (p=0.9)

Table 24: Groups 26 and 27 - Two-colour flow cytometric PBL analysis following DST + CsA + W3/25 induction therapy for 5 days (DA (PVG). Values are mean (SEM.

5.2. Graft histology:

Grafts harvested at 14 days post-transplantation in the DST monotherapy groups showed complete rejection and were replaced by fibrous tissue in both groups (11 and 23). Lymphocytes were still present within the fibrous tissue and at the kidney graft interface (figures 68 and 79).

The grafts harvested from the DST, CsA and W3/25 induction therapy groups (groups 14,15, 26 and 27) showed excellent graft survival and development, especially, of the islets of Langerhans at 14 and 30 days post-transplantation. Graft infiltrates were minimal (figures 72, 73, 75 and 76). Immunocytochemistry showed the islets mostly consisted of insulin positive cells with some peripheral glucagon positive cells and the occasional somatostatin positive cell (figures 77, 86, 78 and 79).

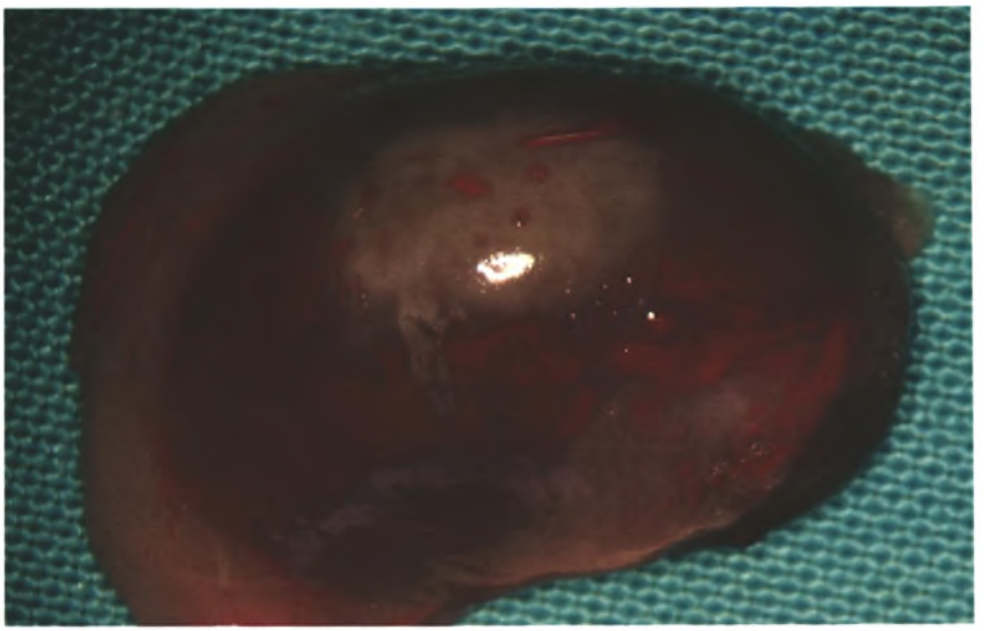

Figure 67: Group 11– A photomicrograph of FRPT 14 days post-transplantation (allografts) showing a white fibrous area at the transplantation site. No viable graft is macroscopically visible.

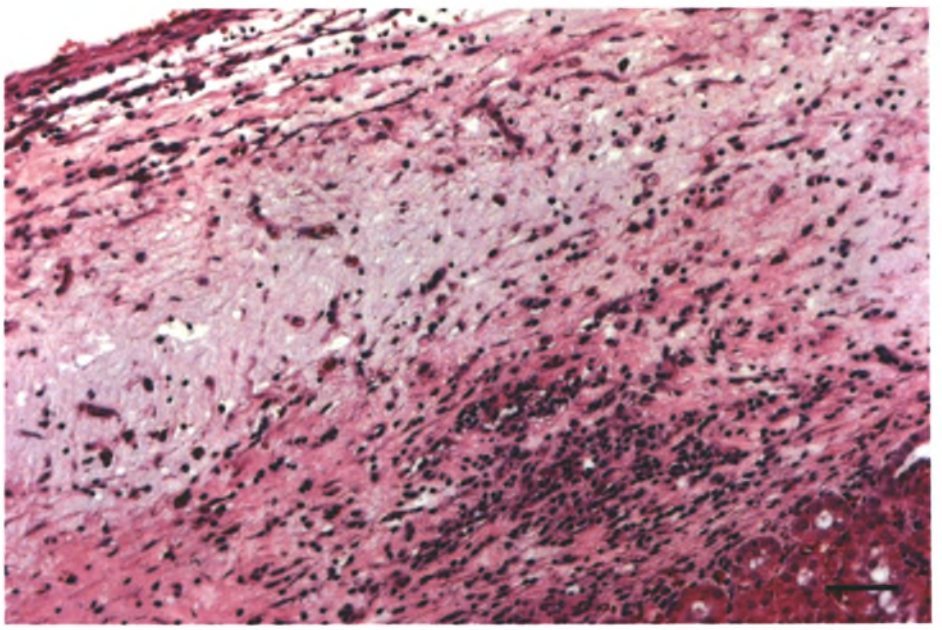

Figure 68: Group 11 – DST monotherapy shows advanced acute rejection with complete destruction of the grafts that are replaced with fibrous scar tissue. A moderate lymphocytic infiltrate is present at the site of engraftment (H&E x100). Scale bar = 90 (m.

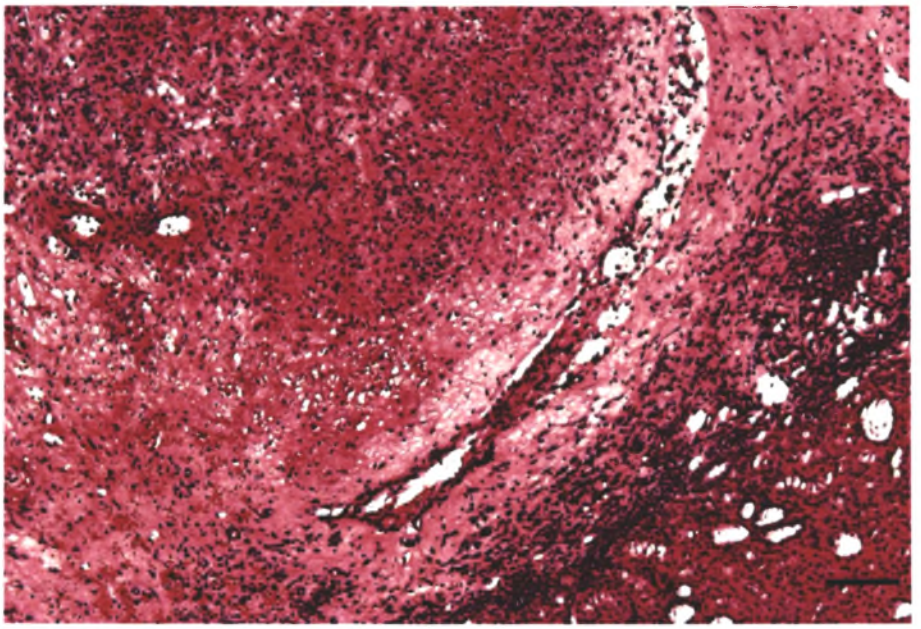

Figure 69: Group 12 – DST and CsA induction therapy resulted in accelerated rejection of the grafts at 14 days post-transplantation. A dense band of lymphocytes are present at the kidney graft interface. The grafts are completely destroyed. An eosinophilic fibrinoid exudate is seen at the graft site (H&E x50). Scale bar = 170 μm.

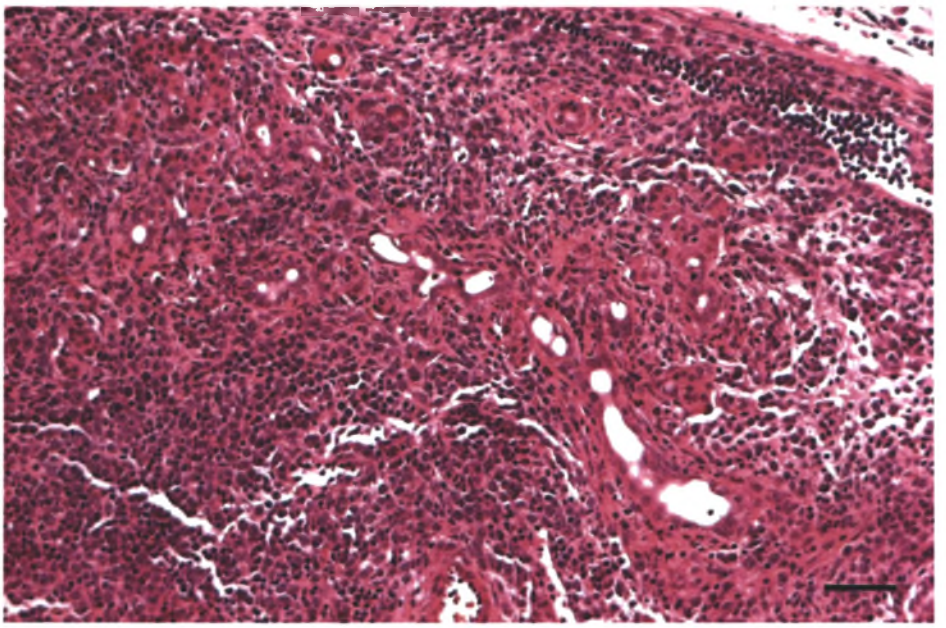

Figure 70: Group 13 – DST and W3/25 combination therapy, 14 days post-transplantation shows "surviving" identifiable graft with islets and exocrine tissue present within a heavy lymphocytic graft infiltrate (H&E x50). Scale bar = 170 μm.

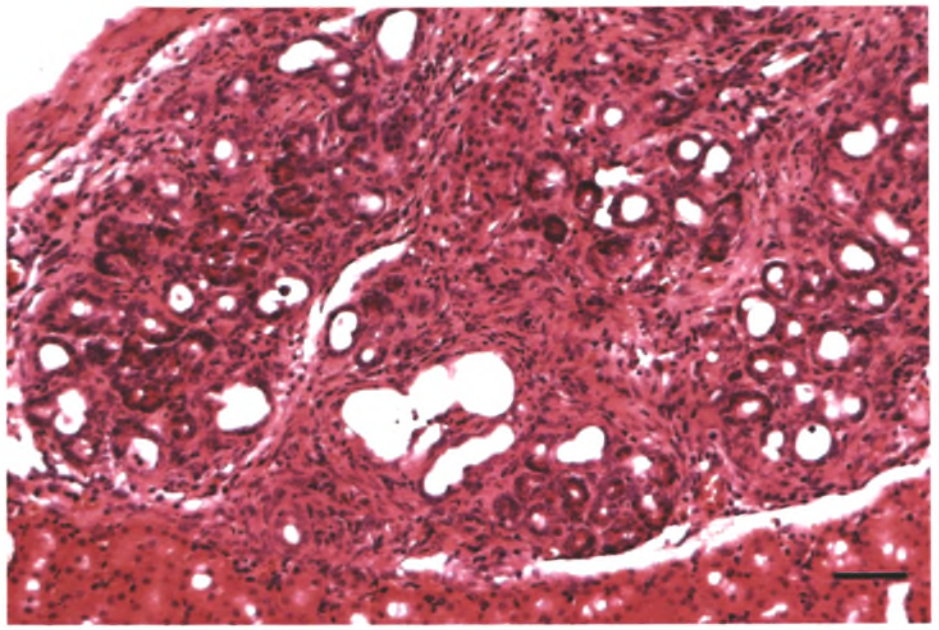

Figure 71: Group 14 – DST, CsA and W3/25 combination therapy, 14 days post-transplantation shows excellent graft survival with islets, and dilated exocrine ducts. Very little graft infiltrate by lymphocytes is present (H&E x50). Scale bar = 170 μm.

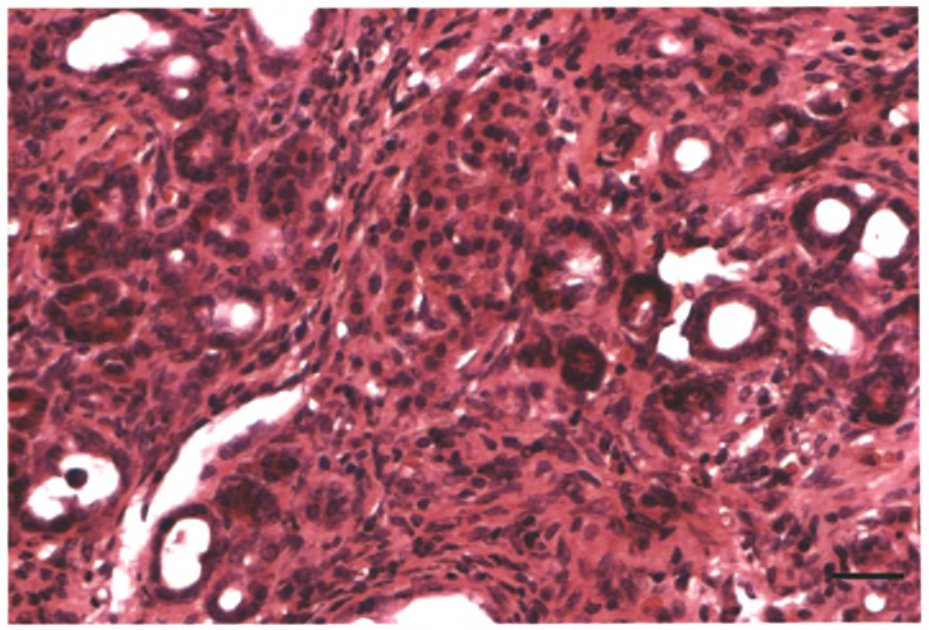

Figure 72: Group 14 – Higher magnification shows an islets and surrounding exocrine tissue. The exocrine tissue shows signs of atrophy and dilatation of ducts. Almost no lymphocytic graft infiltrate is present (H&E x100). Scale bar = 20 μm.

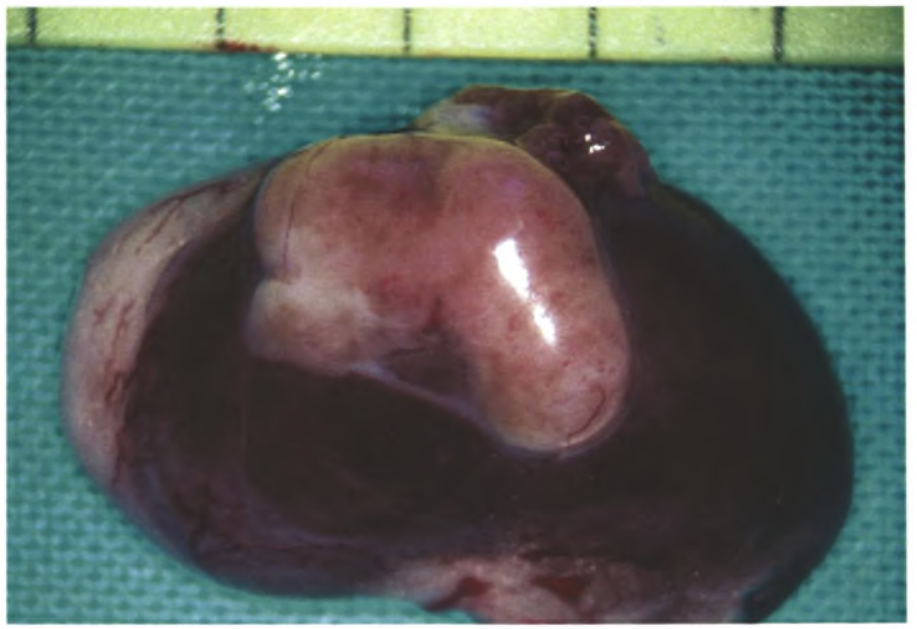

Figure 73: Group 15 – A photomicrograph of FRPT 30 days post-transplantation shows the presence of large coalesced subcapsular mass representing proliferating FRP allografts.

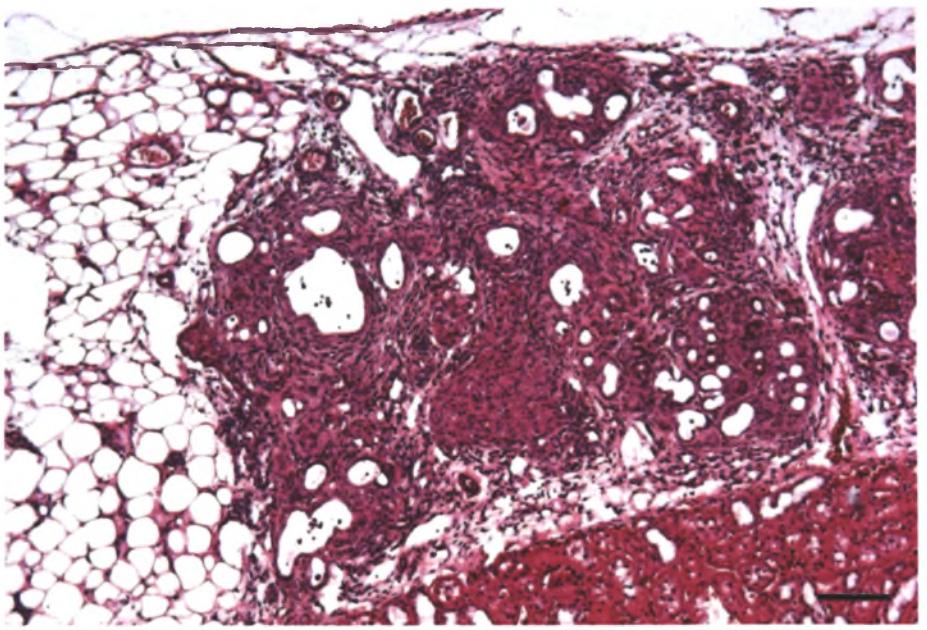

Figure 74: Group 15 – DST, CsA and W3/25 combination therapy, 30 days post-transplantation shows viable graft with islets, and dilated exocrine ducts. Perigraft subcapsular fat (WAT) is present (H&E x50). Scale bar = 170 μm.

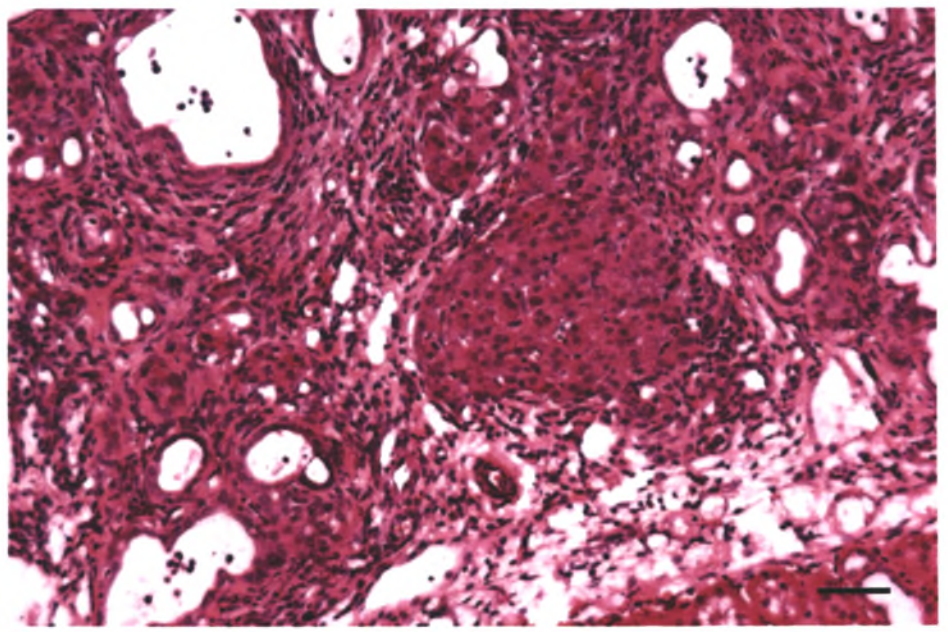

Figure 75: Group 15 – Higher magnification shows well-developed islets with a moderate peri-graft lymphocytic infiltrate and dilated atrophing exocrine ducts and acini (H&E x100). Scale bar = 90 μm.

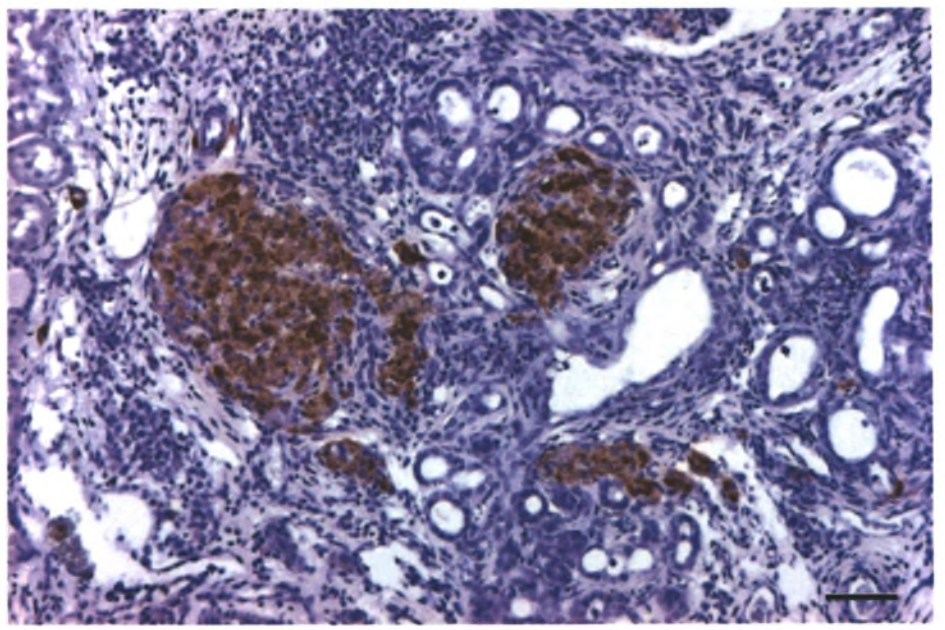

Figure 76: Group 15 – ICC to demonstrate insulin shows that the islets consist mainly of insulin positive cells (Insulin x 100). Scale bar = 90 μm.

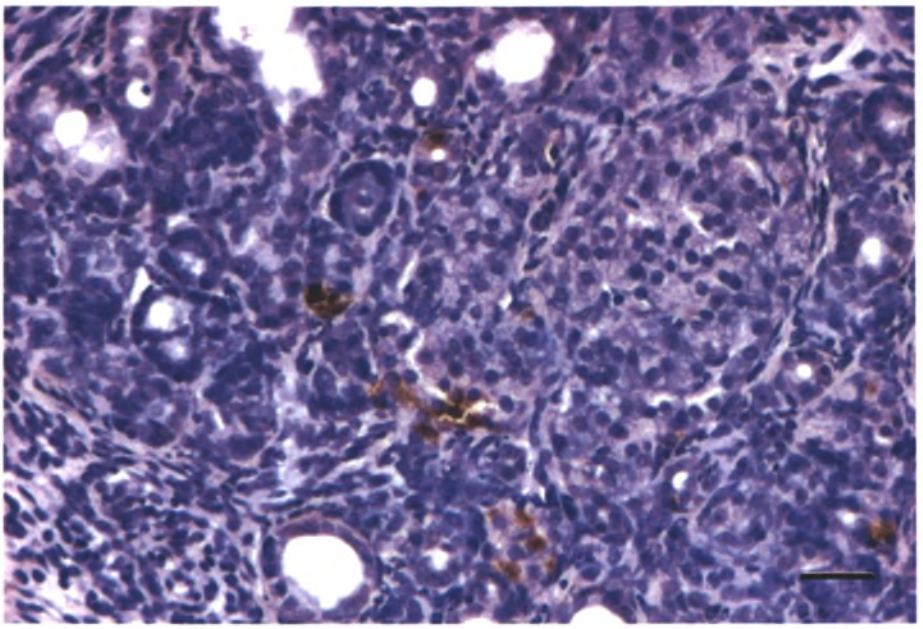

Figure 77: Group 15 – ICC to demonstrate glucagon shows a few glucagon positive cells mainly limited to the periphery of the islets. The occasional glucagon positive ductular cells as is demonstrated in this photomicrograph were an interesting observation (Glucagon x 200). Scale bar = 40 μm.

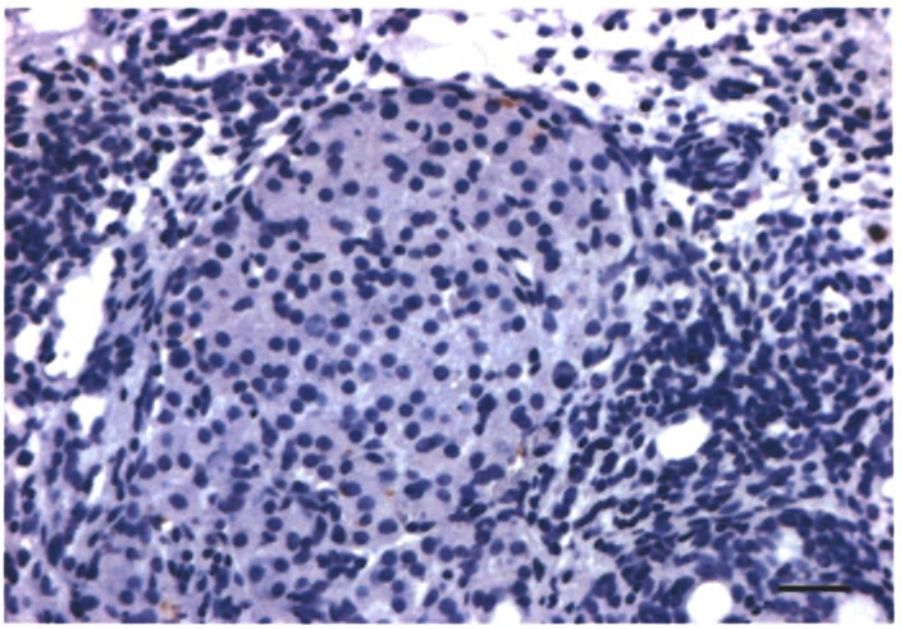

Figure 78: Group 15 – ICC to demonstrate somatostatin shows the occasional somatostatin positive cells in the periphery of the islets. Somatostatin positive cells, if present, were limited to 1 – 2 cells per islet (Somatostatin x 200). Scale bar = 40 μm.

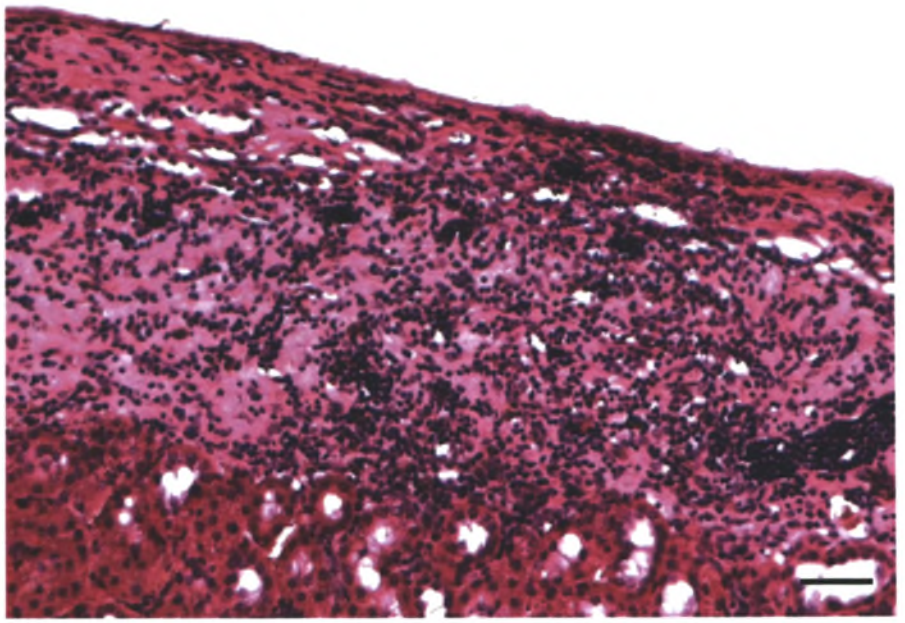

Figure 79: Group 25 - DST only shows a presensitized hyperacute type of rejection. The grafts are completely destroyed. A heavy lymphocytic infiltrate is still present (H&E x100). Scale bar = 90 μm.

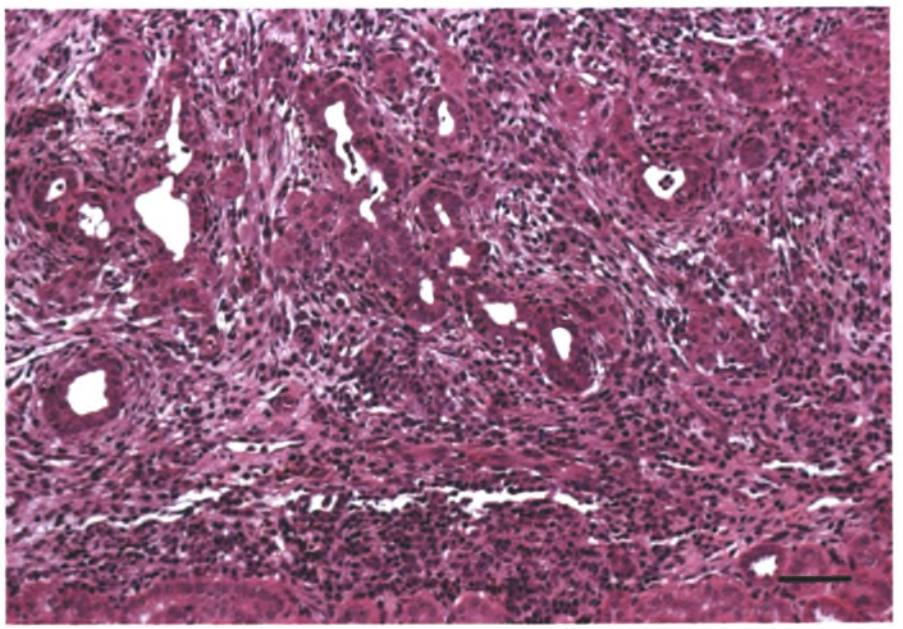

Figure 80: Group 26 - DST and CsA combination therapy showed some graft preservation albeit within a heavy lymphocytic infiltrate (H&E x100). Scale bar = 90 μm.

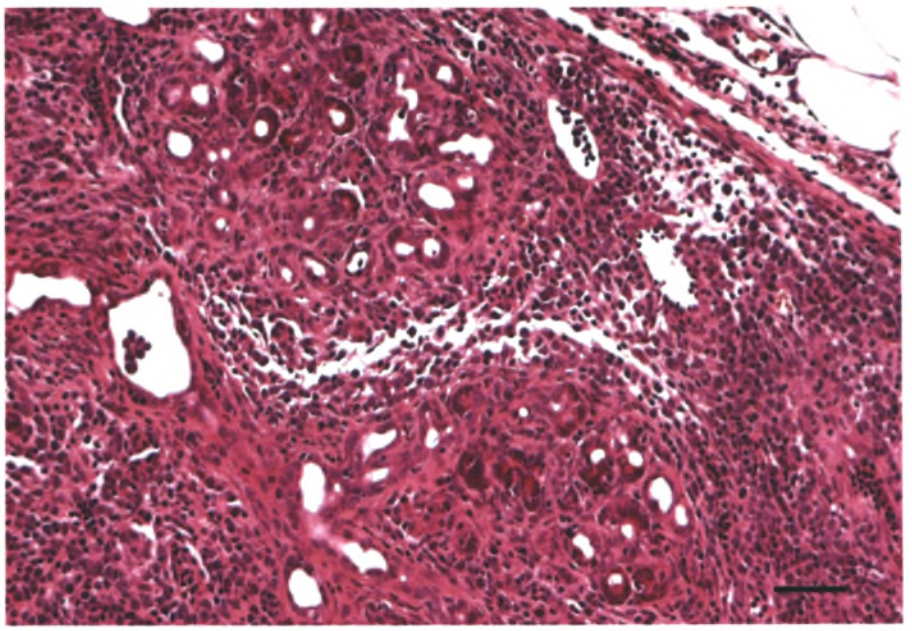

Figure 81: Group 27 - DST and W3/25 combination therapy showed graft survival and the presence of a heavy lymphocytic infiltrate (H&E x100). Scale bar = 90 μm.

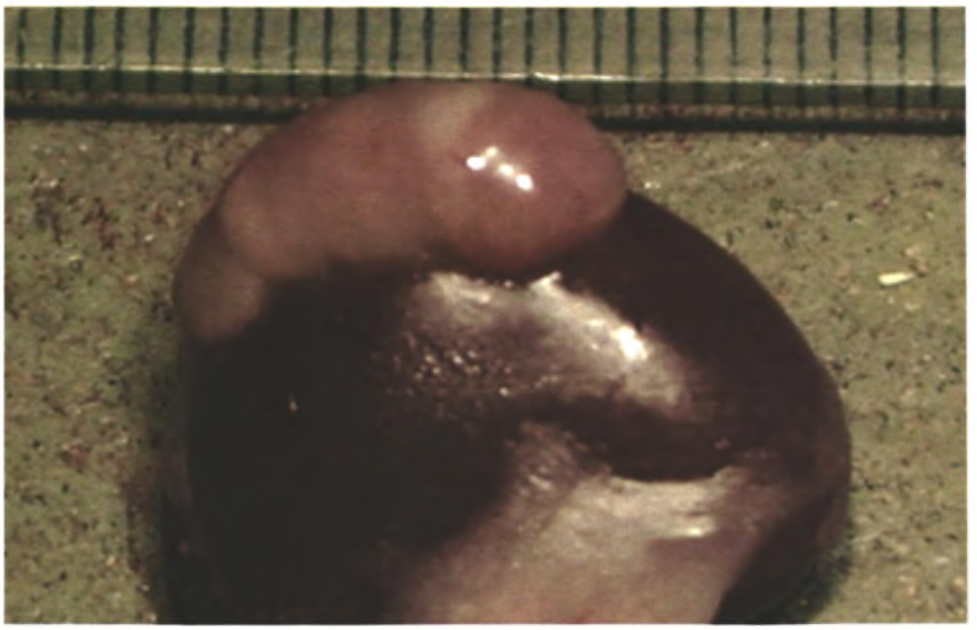

Figure 82: Group 16– A macroscopic view of FRPT 30 days post-transplantation. The presence of the grafts and formation of fat pads, as a result of insulin secretion, is clearly visible beneath the kidney capsule.

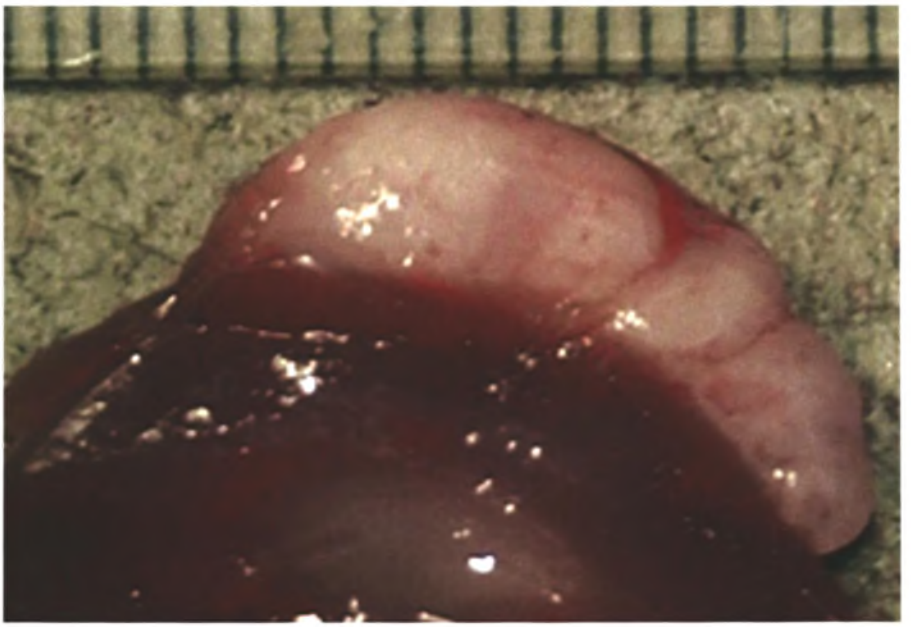

Figure 83: Group 16– A cross section of the subcapsular grafts following FRPT 30 days post-transplantation shows coalescence of the grafts under the kidney capsule.

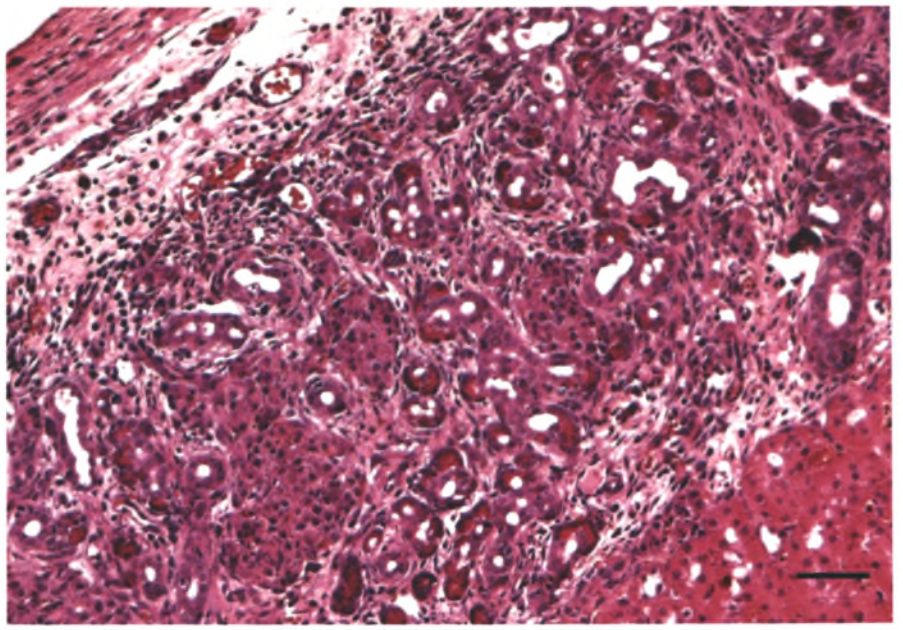

Figure 84: Group 26– DST, CsA and W3/25 combination therapy, 14 days post-transplantation, shows well-preserved grafts and islets. A mild lymphocytic infiltrate is present (H&E x100). Scale bar = 90 μm.

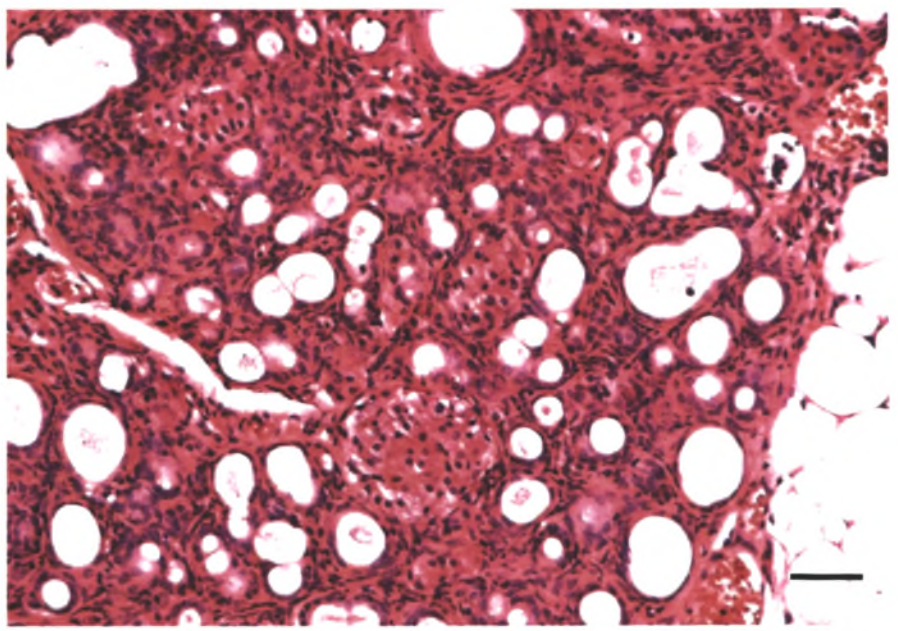

Figure 85: Group 27 – DST, CsA and W3/25 combination therapy, 30 days post-transplantation shows well-developed islets and atrophing exocrine tissue and fat formation. A moderate lymphocytic infiltrate is present in the graft (H&E x100). Scale bar = 90 μm.

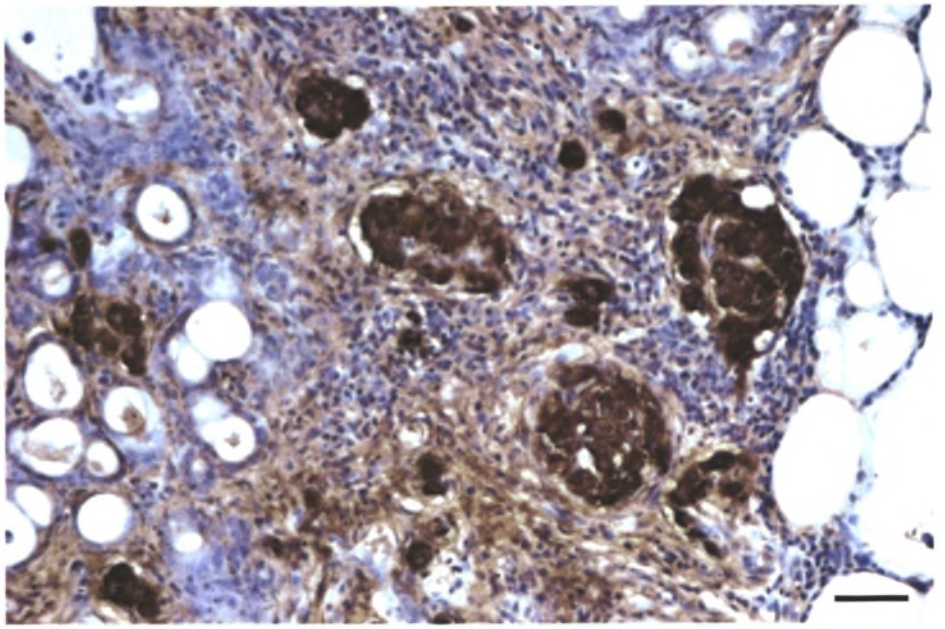

Figure 86: Group 27 – DST, CsA and W3/25 combination therapy, 30 days post-transplantation. ICC to demonstrate insulin shows insulin positive islets of various sizes scattered throughout the graft (Insulin x 50). Scale bar = 170 μm.

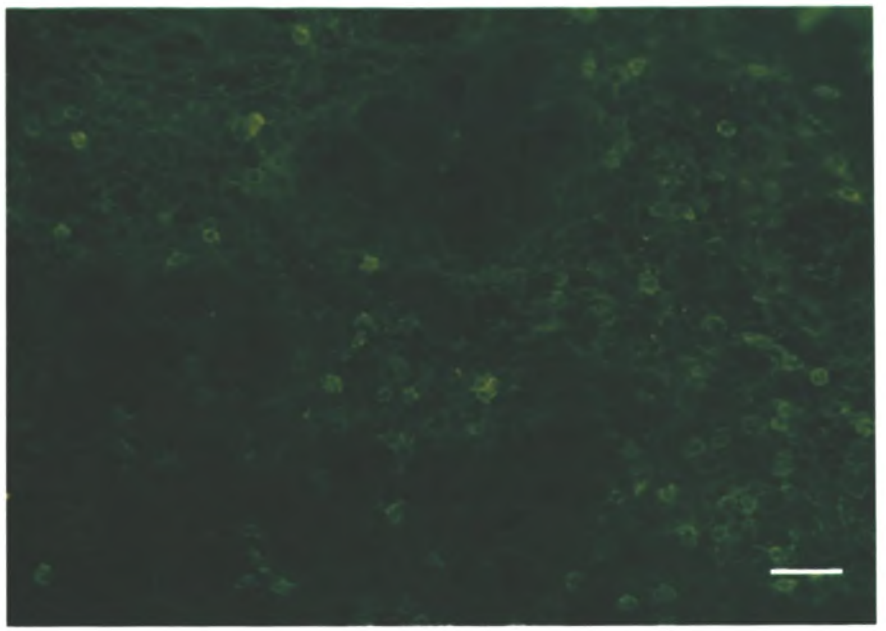

Figure 87: Group 27 – Immunofluorescence to demonstrate $CD4^+$ lymphocyte infiltrates in the graft 30 days post-transplantation. The presence of a light to mild perigraft infiltrate, in which CD4+ cells predominate (CD4/CD8 ratio 1.5), was a consistent finding following DST, CsA and W3/25 combination therapy (CD4 x 300). Scale bar = 30 μm.

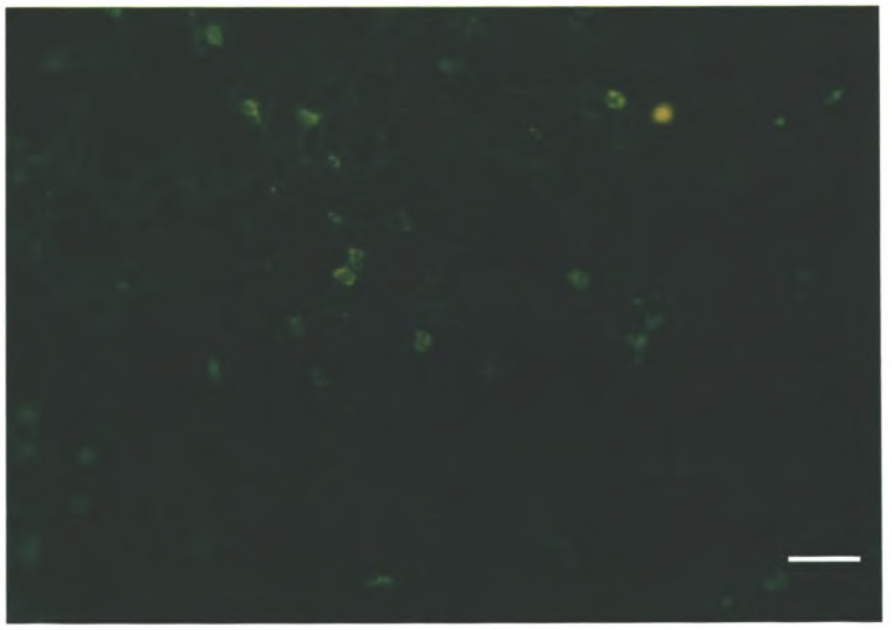

Figure 88: Group 27 – Immunofluorescence to demonstrating $CD8^+$ lymphocyte infiltrates in the graft 30 days post-transplantation. (CD8 x 300). Scale bar = 30 μm.

SECTION 6.

RESULTS DIABETIC GROUPS – REVERSAL OF STREPTOZOTOCIN (STZ) INDUCED DIABETES BY AUTOLOGOUS AND ALLOGENEIC FOETAL RAT PANCREATIC TRANSPLANTATION (FRPT).

Results in this section confirms the efficacy of autologous FRPT in reversing STZ induced diabetes (group 32) but, in addition, demonstrates allogeneic reversal of STZ induced diabetes (group 33) using an immunemodulation strategy that includes DST, CsA and W3/25 induction therapy. This strategy results in donor specific graft unresponsiveness without the need for daily immunosuppression.

Autologous and allogeneic FRPT (groups 32 and 33) prevented the significant weight loss and reversed the polydipsia and polyuria and restored normoglycaemia that occurred in the non-transplanted controls (Tables 25 and 29).

IVGTT showed that, following autologous and allogeneic FRPT (groups 32 and 33), animals were able to respond to a 0.5 g/kg glucose bolus while the non-transplanted diabetic controls showed no response (Tables 26, 28 and figures 91, 99).

At autopsy, the grafts present in both the autologous and allogeneic groups (groups 32 and 33) appeared as prominent white sub-capsular masses associated with fat (figures 92, 100 and 101).

Histology of the harvested auto- and allografts (groups 32, 33), 30 days post-transplantation, confirmed the presence of islets of Langerhans with a normal appearance (Figures 93, 102 and 103).

Immunocytochemistry showed insulin positive islets scattered throughout the grafts (figures 94, 104 and 105) and some glucagon, somatostatin and pancreatic polypeptide positive cells (figures 95, 96, 97 and 106).

The ultrastructure of the islets following autologous and allogeneic FRPT (groups 32 and 33) confirmed the presence of viable and well granulated endocrine cells containing the characteristic insulin-like granules (figures 998 and 107).

Morphometry to quantify the islet size, the % β- and α-cell area of the islets and the β-cell / α-cell ratio of the grafts (tables 26 and 29) gives an objective comparison of the graft islet morphometry (groups 32 and 33) with the normal (groups 28, 29) and diabetic pancreatic islets (groups 30 and 31).

6.1. STREPTOZOTOCIN (STZ) INDUCED DIABETES:

The effect a single 50 mg/kg IV injection of STZ, a diabetogenic agent, on the insulin positive cells in the islets of Langerhans is clearly demonstrated in Figures 89 and 90.

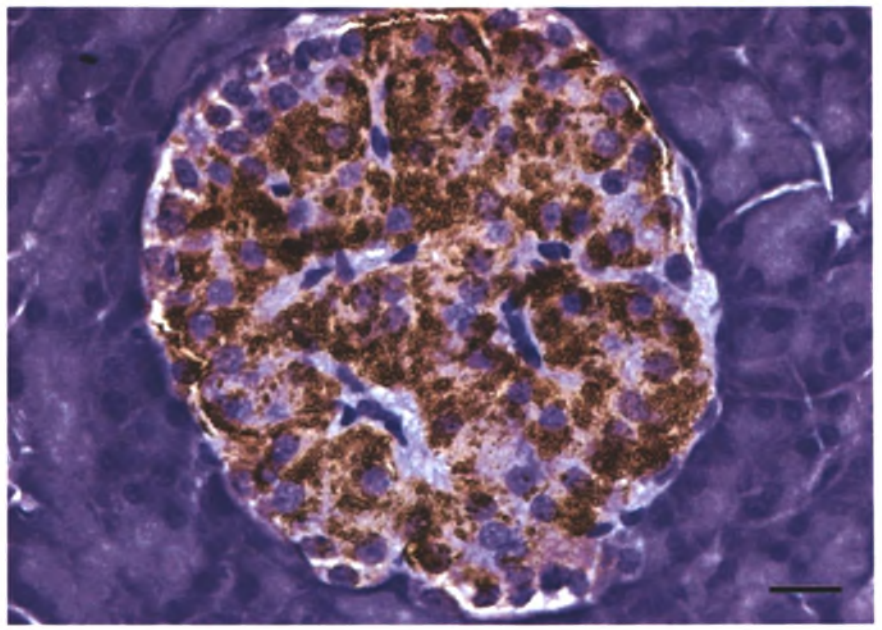

Figure 89: Group 29 - Immunocytochemistry demonstrating insulin positive staining of the majority of cells within in the islet of Langerhans in a normal non-diabetic SD rat (insulin x400). Scale bar = 20 μm.

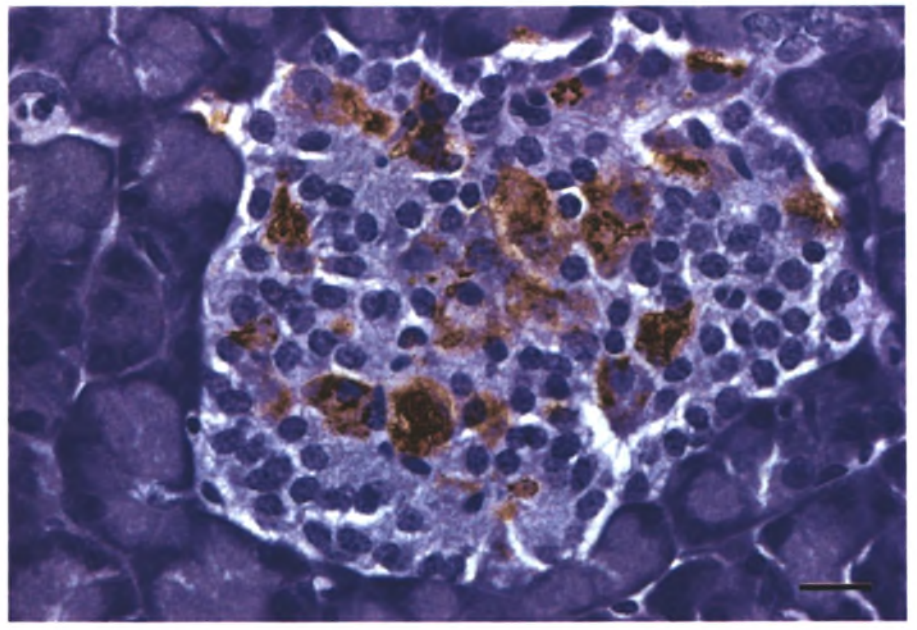

Figure 90: Group 31 - Immunocytochemistry demonstrating the severe reduction of insulin positive staining within the islet of Langerhans 30 days after the induction of diabetes by a single 50 mg/kg STZ intravenous injection (insulin x400). Scale bar = 20 μm.

6.1. WAG Autologous Diabetic Transplantation Groups.

Group	Body weight (g)	WBG mmol/l	Water intake ml/24hrs	Urine output ml/24hrs
Group 28	344 ± 12.0	6.42 ± 0.1	21.3 ± 6.8	7.9 ± 1.3
Group 30	249 ± 8.0 (p= 0.005)	20.84 ± 1.3 (p=0.002)	55.8 ± 10.9 (p = 0.002)	29.9 ± 6.4 (p = 0.008)
Group 32	325 ± 12.1 (p= 0.4)	6.37 ± 1.0 (p=0.4)	19.0 ± 10.7 (p = 0.6)	7.5 ± 3.4 (p = 0.7)

Table 25: Autologous transplantation diabetic experimental animal weights and metabolic profiles in normal non-diabetic controls (group 28), the diabetic non-transplanted controls (group 30) and the autologous transplantation diabetic experimental animals (group 32).

Time	Group 28	Group 30	Group 32
Basal	6.29 ± 0.17	20.84 ± 1.3	7.9 ± 0.13
1 min	18.91 ± 1.16	27.7 ± 3.1	19.63 ± 1.39
2 min	16.24 ± 0.98	25.36 ± 2.44	18.65 ± 1.29
3 min	17.3 ± .7	23.42 ± 4.38	17.35 ± 0.964
5 min	15.3 ± 0.58	27.56 ± 5.24	14.5 ± 2.14
10 min	12.4 ± 0.81	25.64 ± 2.16	13.5 ± 0.77
20 min	8.53 ± 0.57	25.34 ± 1.22	10.85 ± 0.6
30 min	6.39 ± 0.47	25.62 ± 2.81	8.58 ± 0.21
40 min	6.2 ± 0.48	25.36 ± 1.2	7.47 ± 0.49
50 min	5.46 ± 0.57	24.46 ± 1.51	6.15 ± 0.25
60 min	6.9 ± 0.77	23.88 ± 1.57	5.6 ± 0.2

Table 26: Whole Blood Glucose values of the normal non diabetic controls (group 28), the diabetic non-transplanted controls (group 30) and the autologous transplantation diabetic experimental animals (group 32) at various time points during an IVGTT; values are in mmol/l ± SEM.

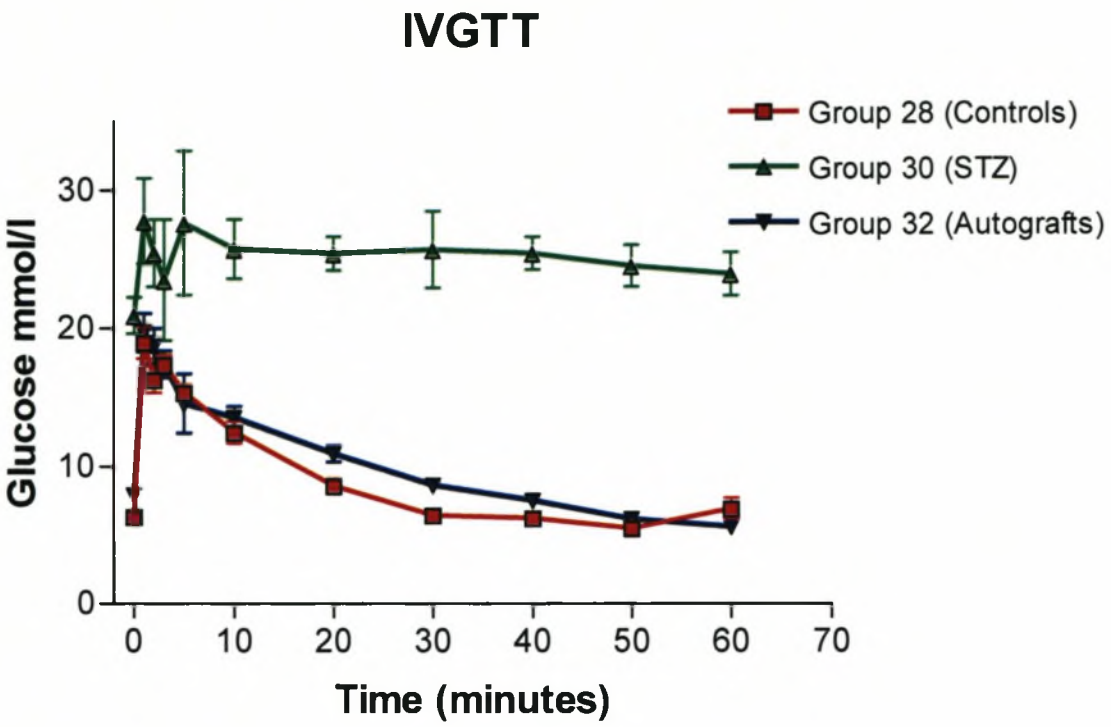

Figure 91: Groups 28, 30, 32 - IVGTT graph comparing the WBG response to an intravenous glucose bolus at different time points in the syngeneic transplantation groups. The IVGTT values showed no significant differences between the syngeneic group and the normal controls (group 28 vs. 32; p=0.6), while the diabetic controls differed very significantly from the normal controls (group 30 vs. group 28 p =< 0.0001).

	AUC (mm^2)	% of normal AUC	K-value
Normal controls	511.1	100	2.31
Diabetic controls	1515	296	<1
Group 32	578.8	113	1.955

Table 27: Area under the curve- (AUC) and K-values following IVGTT in the diabetic and syngeneic transplantation groups.

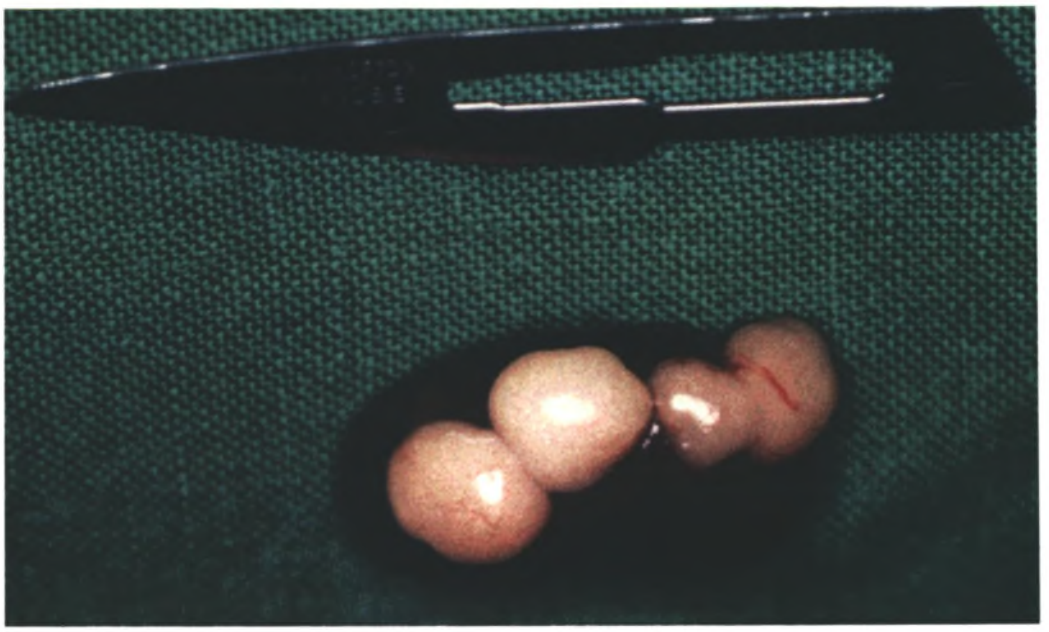

Figure 92: Group 32 - FRPT grafts, 1 month post transplantation are clearly visible as discrete fat deposits on the surface of the kidney.

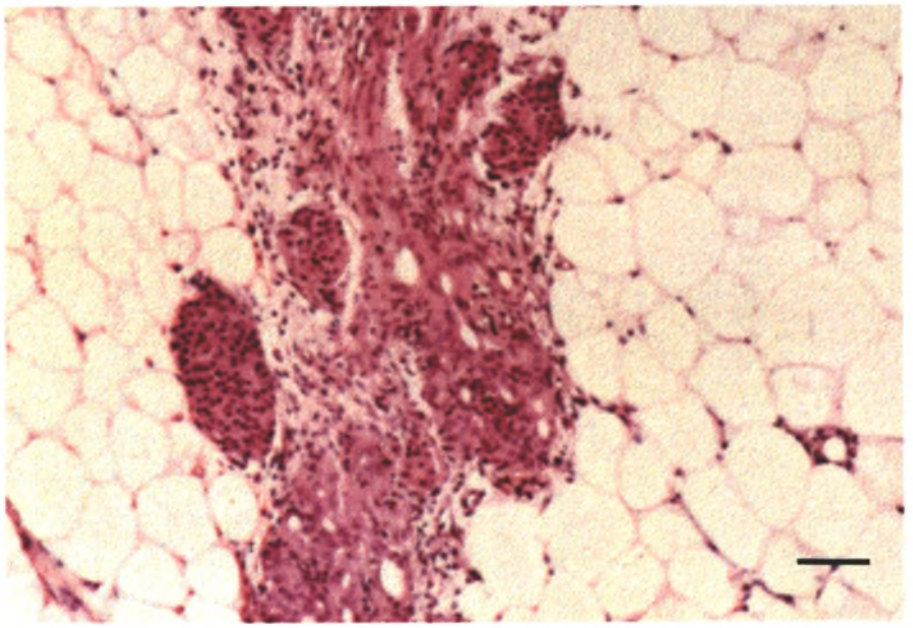

Figure 93: Group 32 - The development of inter- and intragraft adipose tissue (white fat) was a constant feature following successful syngeneic subcapsular foetal rat pancreas transplantation. 30 days post-transplantation grafts consisted of well-developed islets within fibrous tissue or in surrounding adipose tissue. The aethiology of the fat deposition remains unknown. Exocrine tissue is absent H&E x100. Scale bar = 90 μm.

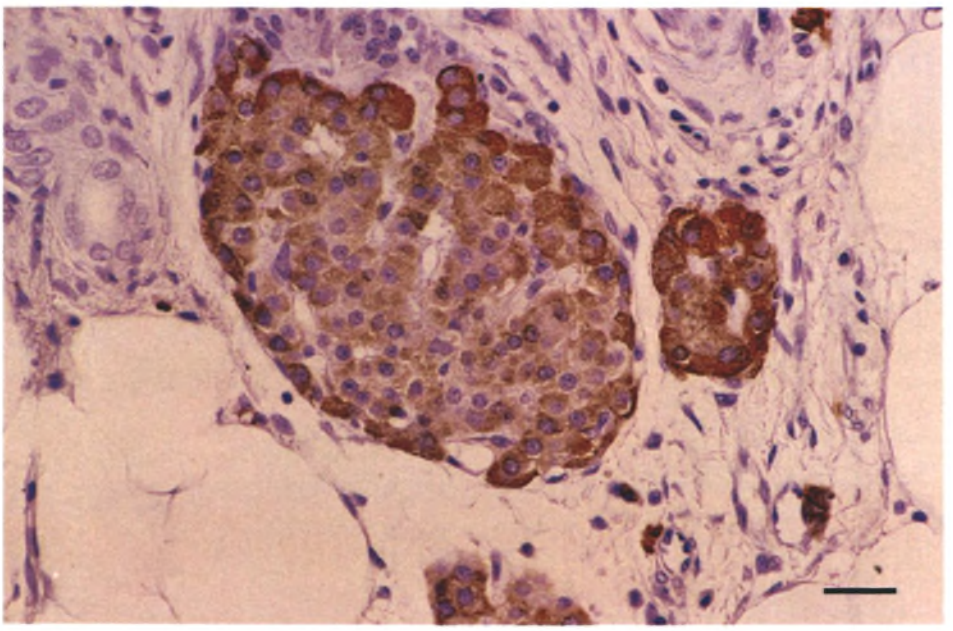

Figure 94: Group 32 (autografts) - Immunocytochemistry of an islet of Langerhans, 30 days post-transplantation, shows the islets consisted mostly of insulin positive cells (x.400). Scale bar = 20 µm.

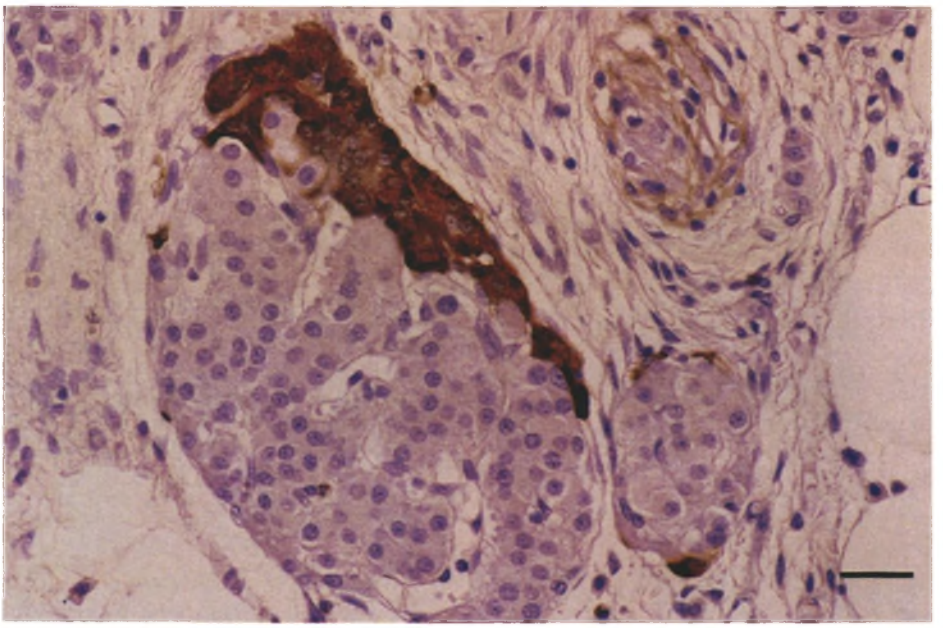

Figure 95: Group 32 (isografts) - Glucagon positive cells formed an incomplete band at the marginal zone of the islet (x400). Scale bar = 20 µm.

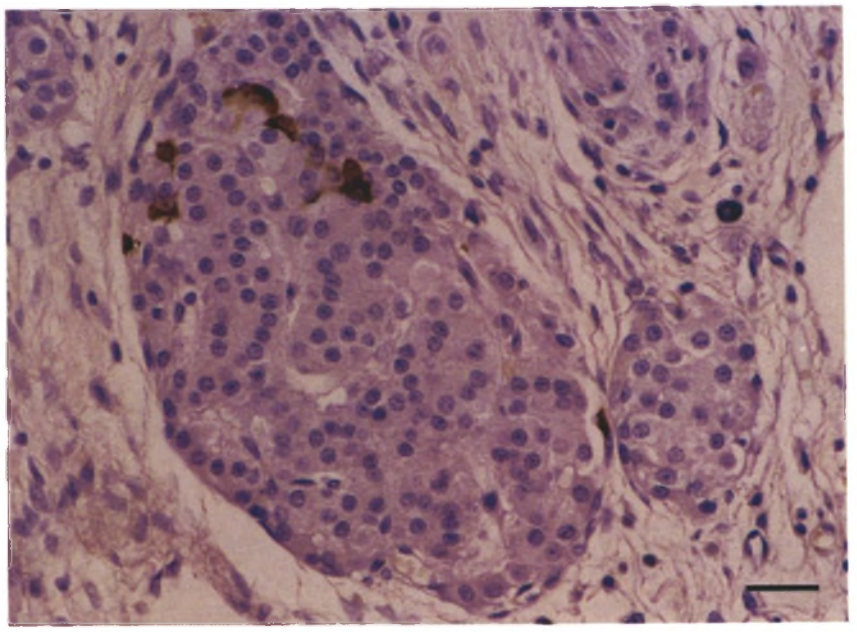

Figure 96: Group 32 - A few somatostatin positive cells are present in the marginal zone of the islet (somatostatin x400). Scale bar = 20 μm.

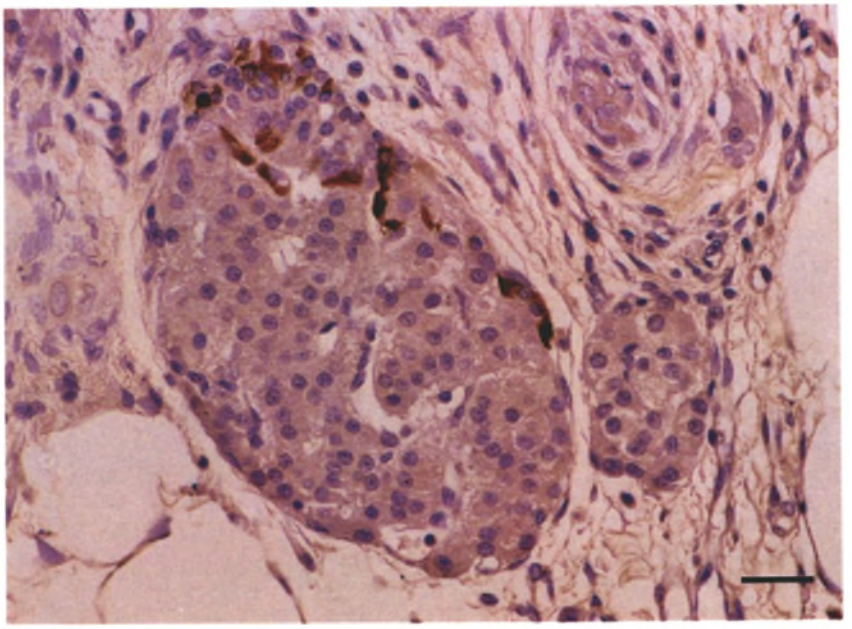

Figure 97: Group 32 - Pancreatic polypeptide positive cells are also seen in the marginal zone of the islet (PP x400). Scale bar = 20 μm.

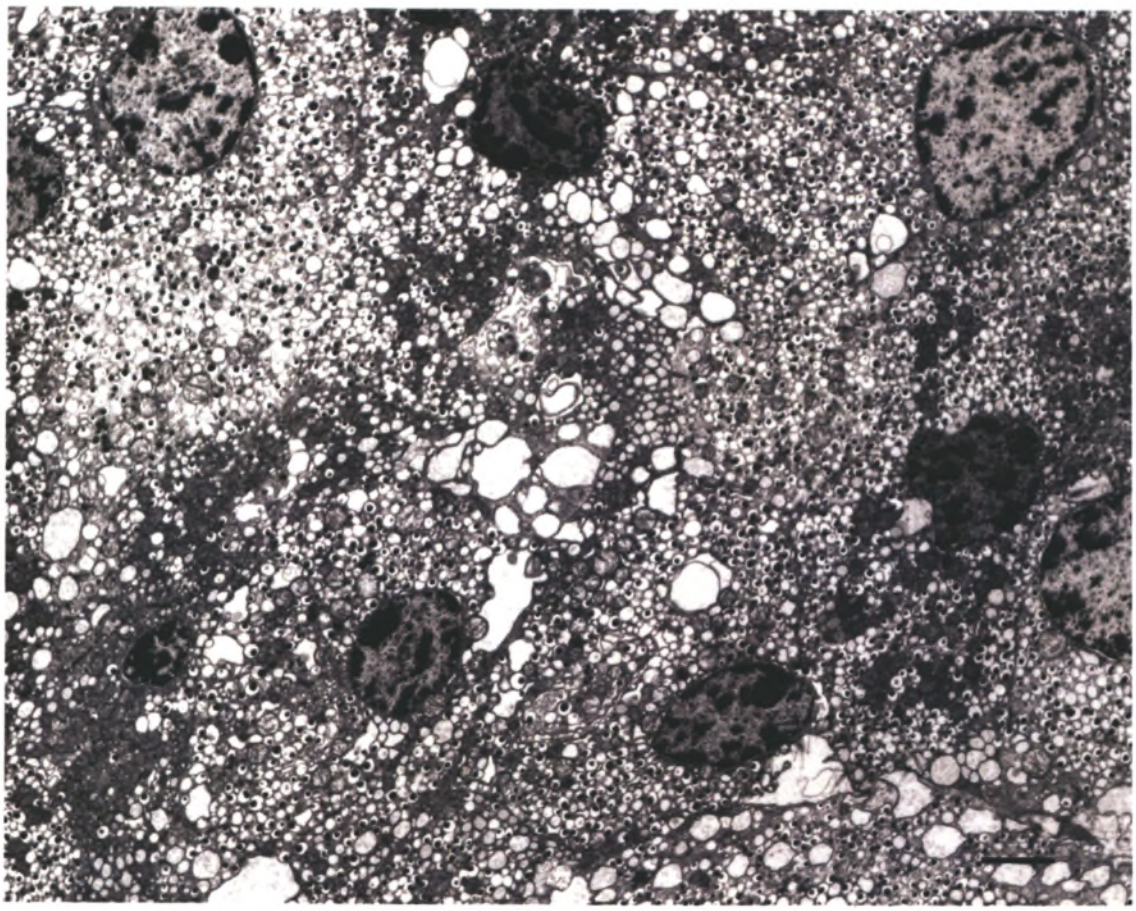

Figure 98: Group 32 - Electron micrograph of the central part of an islet of Langerhans, 30 days post-transplantation shows well-granulated endocrine cells containing characteristic insulin granules (x4500). Scale bar = 2.2 μm.

	Group 28	Group 30	Group 32
% β-cell area/islet area (p vs. control)	64.14 ± 1.94	28.4 ± 12.48 (p=0.01)	57.12 ± 6.2 (p=0.3)
β-cell size (group vs. control)	158.43 ± 8.16	66.25 ± 6.77 (p=0.01)	123.78 ± 20.01 (p=0.2)
β-cell/α-cell (group vs. control)	2.24 ± 0.16	0.44 ± 0.26 (p=0.01)	17.4 ± 1.9 (p=0.01)

Table 28: Groups 28, 30 and 32 - Morphometric analysis of the islets of Langerhans in the STZ autologous transplantation groups.

6.2. Allogeneic Diabetic Transplantation Groups

Group	Body Weight (g)	WBG mmol/l	Water intake ml/24hrs	Urine output ml/24hrs
Group 29	390.2 ± 24.1	6.36 ± 0.5	28 ± 2.16	10.2 ± 2.11
Group 31	324.8 ± 19.9 (p=0.03)	14.08 ± 3.2 (p=0.008)	50.6 ± 11.89 (p=0.06)	38.4 ± 10.74 (p=0.05)
Group 33	406 ± 12.8 (p=0.2)	6.9 ± 1.9 (p=0.5)	34.8 ± 3.23 (p=0.1)	18.6 ± 5.73 (p=0.4)

Table 29: Body weight and metabolic profiles in normal non-diabetic SD controls (group 29), the diabetic non-transplanted SD controls (group 31) and the DA⇒SD allogeneic transplantation diabetic experimental animals (group 33).

Time (min)	Group 29	Group 31	Group 33
0	6.36 ± 0.49	14.4 ± 3.54	7.57 ± 1.58
1	16.58 ± 2.59	21.78 ± 4.13	16.84 ± 3.34
3	16.12 ± 0.54	24.5 ± 3.25	15.01 ± 1.56
5	14.6 ± 1.2	24.92 ± 3.26	15.07 ± 1.39
10	13.22 ± 0.65	23.08 ± 3.52	15.17 ± 1.69
15	12.34 ± 0.64	21.56 ± 3.24	14.7 ± 2.19
30	9.9 ± 0.7	22.54 ± 3.98	12.9 ± 2.47
60	6.5 ± 0.63	19.06 ± 3.57	10.14 ± 2.64

Table 30: Groups 29, 31 and 33 - Intravenous glucose tolerance test values following injection of a single bolus of 0.5 g/kg dextrose solution in the allogeneic diabetic groups; results are mean mmol/l values ± SEM.

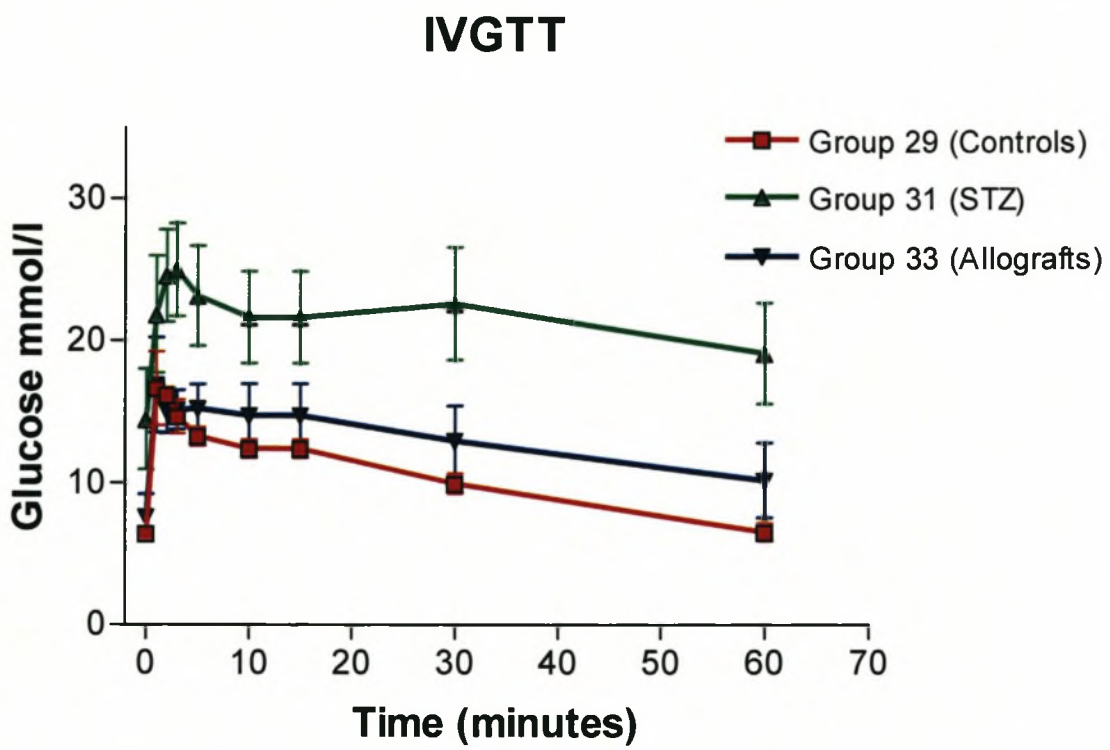

Figure 99: Groups 29, 31, 33 - IVGTT graph of the allogeneic diabetic groups, illustrating the WBG values at different time points during the glucose tolerance test, following injection of a

difference between the normal controls (group 29) and the allogeneic diabetic transplantation group (group 33) p=0.4, while there was a significant difference between the normal controls and the diabetic controls (group 31) p=0.0002.

	AUC (mm^2)	% of normal AUC	K-value
Normal controls	621.1	100	2.175
Diabetic controls	1559	251	<1
Group 33	777	125	1.495

Table 31: Area under the curve- (AUC) and K-values following IVGTT in the diabetic and allogeneic diabetic transplantation groups.

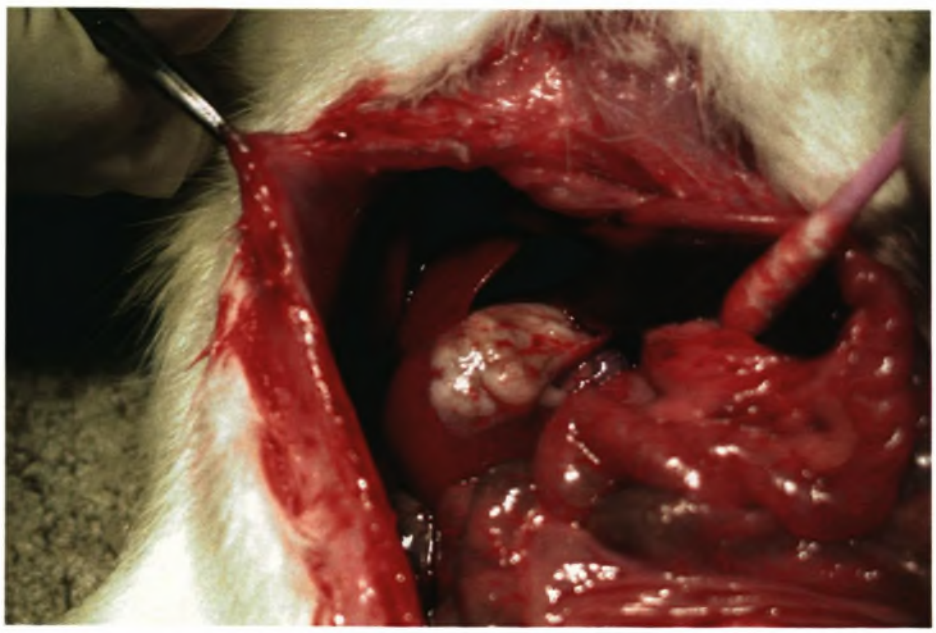

Figure 100: Group 33 - Macroscopic photomicrograph of an exposed kidney 30 days post-FRPT (allotransplantation) following DST, CsA and W3/25 induction therapy, showing the grafts in situ. To reverse STZ induced diabetes, sufficient endocrine mass has to be present (6 - 8 grafts) as is seen in this animal. Scale 1:3.5.

Figure 101: Group 33 - Macroscopic photomicrograph of renal subcapsular grafts in the DST, CsA and W3/25 induction therapy (Allograft) group, 30 days post-transplantation. Grafts are associated with white adipose tissue, which may be the result of ectopic insulin secretion. Enhanced blood supply to the grafts is particularly evident in this graft (Scale 1:4).

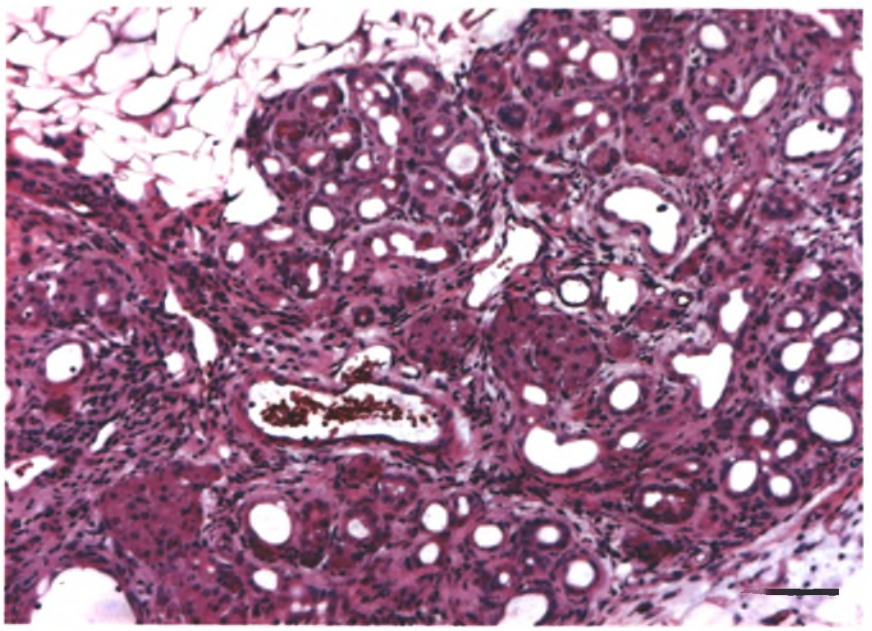

Figure 102: Group 33 - DST, CsA, W3/25 induction therapy resulted in excellent preservation and development of the grafts. Numerous well-developed islets are present. The exocrine component of the grafts consists of dilated ducts and atrophing acinar tissue. Perigraft adipose tissue is present (H&E x 100). Scale bar = 90 μm.

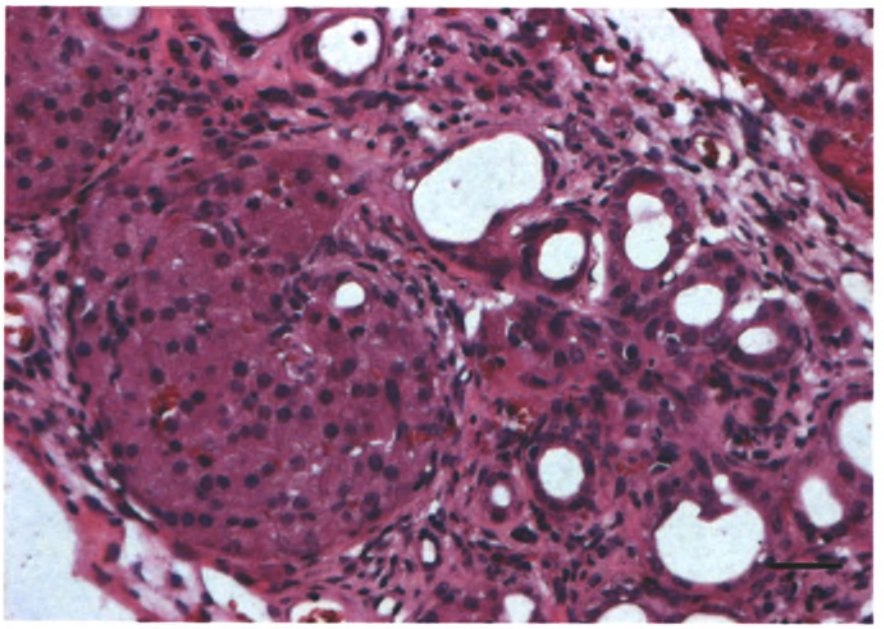

Figure 103: Group 33 (Allograft: post-transpalntation) - higher magnification showing a well-developed islet and some atrophing exocrine tissue components (H&E x 400). Scale bar = 20 μm.

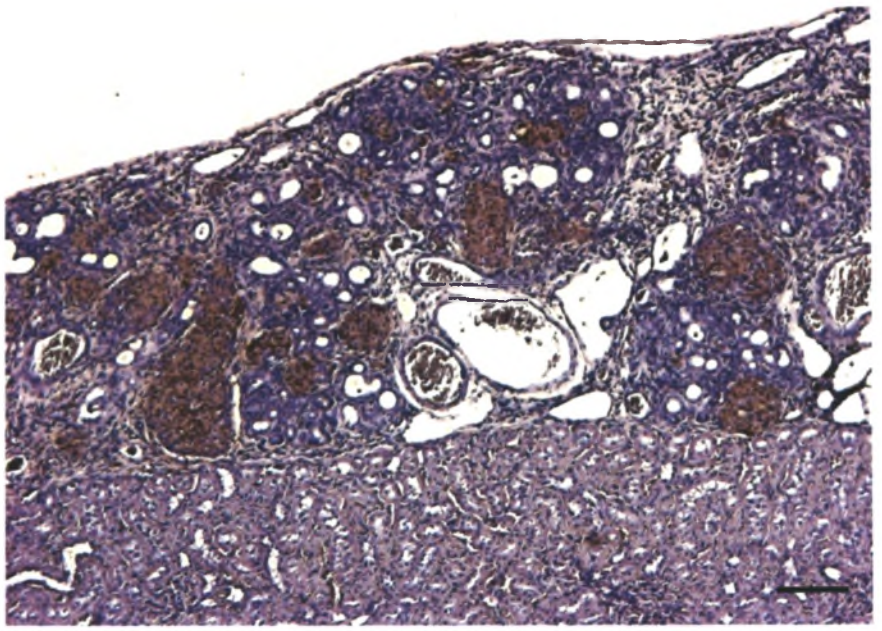

Figure 104: Group 33 - Insulin immunocytochemistry demonstrating the presence of insulin positive (brown staining) islets of Langerhans distributed throughout the allograft (insulin x50). Scale bar = 170 μm.

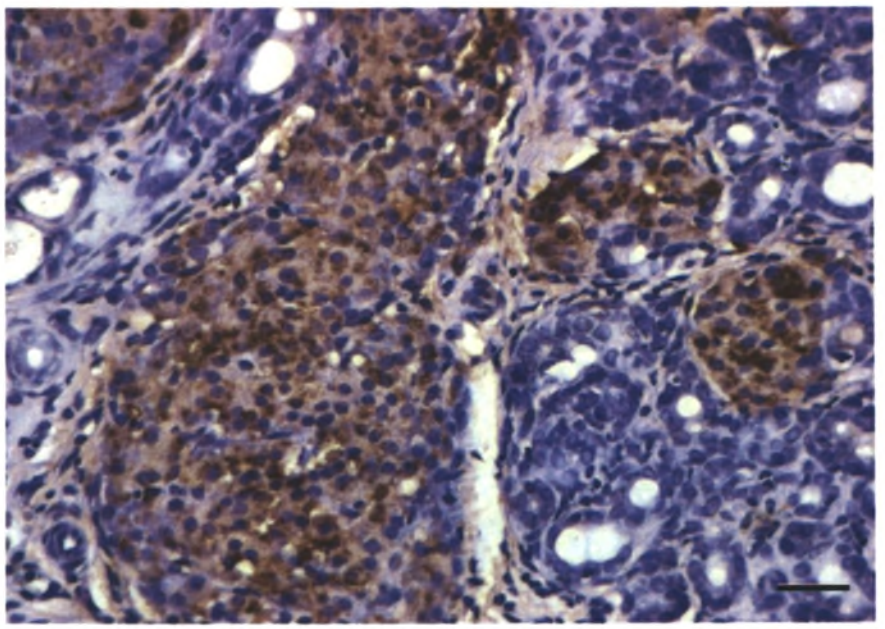

Figure 105: Group 33 - Insulin immunocytochemistry demonstrating insulin in an allografted pancreas demonstrating positively staining within the central portion of the islet (insulin x400). Scale bar = 20 μm.

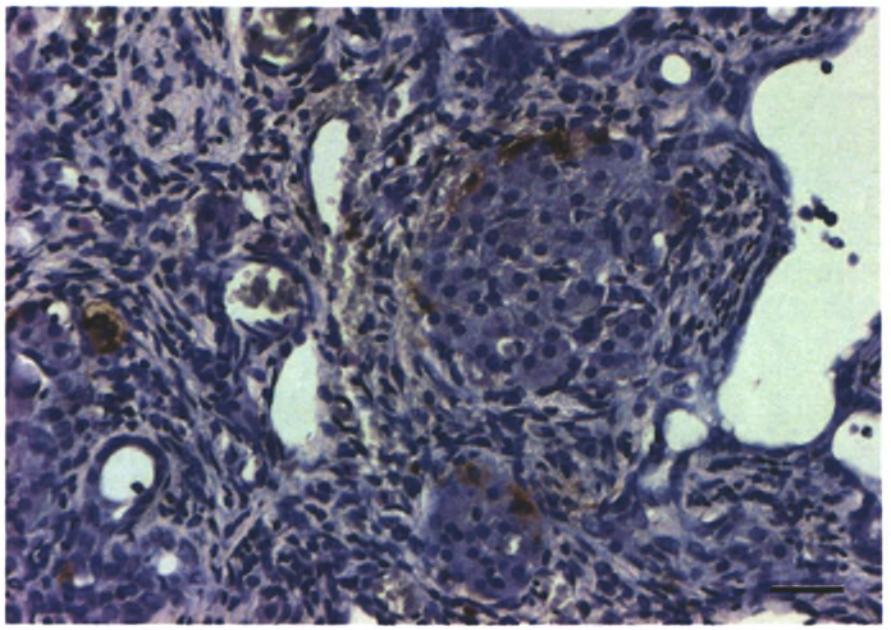

Figure 106: Group 33 - Glucagon immunocytochemistry showed staining of α-cells in the periphery of the islets. Morphometry showed that the % of α-cells was reduced following allogeneic FRPT (glucagon x400). Scale bar = 20 μm.

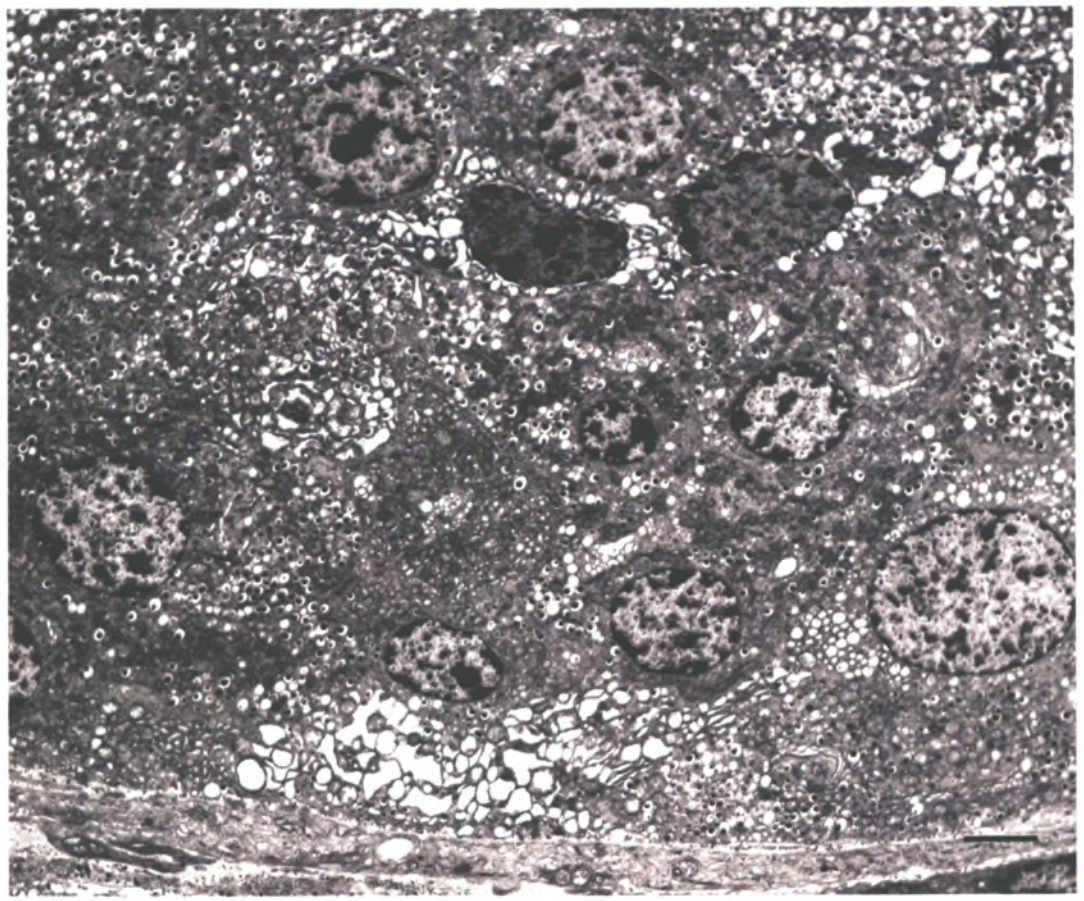

Figure 107: Group 33 - Electron micrograph of a part of an islet of Langerhans 30 days post-transplantation, following DST, W3/25 and CsA induction therapy, shows viable well-granulated endocrine cells containing typical insulin granules (x4500). Scale bar = 2.2 μm.

	Group 29: normal Pancreas (μm^2)	Group 31: STZ Pancreas (μm^2)	Group 33: Allografts (μm^2)
% β - cell area/islet area	54.7 ± 3.10	20.4 ± 2.64 (p=0.003)	39.7 ± 5.65 (p=0.03)
% α - cell area/islet area	21.5 ± 4.54	31.6 ± 3.87 (p=0.1)	3.1 ± 0.81 (p=0.01)
β – cell / α – cell	2.55 ± 0.66	0.72 ± 0.11 (p=0.003)	12.8 ± 2.68 (p=0.02)
Large islet size	82 390 ± 15 478	46 658 ± 6006 (p=0.01)	25 373 ± 4154 (p=0.001)

Table 32: Groups 29, 31, 33 - Morphometrical analysis of insulin and glucagon immunocytochemistry of islets in normal pancreas, STZ pancreas and grafts, following allogenic FRPT. Values are mean ± SEM. P values reflect the difference between the diabetic control pancreata (group 31) and the allogeneic FRP grafts (group 33) compared to the normal non-diabetic control pancreata (group 29).

SECTION 7.

GRAFT HISTOLOGICAL SCORING AFTER AUTOLOGOUS AND ALLOGENEIC TRANSPLANTATION

Group	Graft Scores	Islets	Infiltrate Score	% grafts rejected
Syngeneic Controls (DA ⇒ DA)				
Group 2 Syngeneic	4,3,3,3,3,4,4,3 **(3.4)**	++	0,1,1,1,1,0,0,1 **(0.6)**	0 (0/8)
Allografts (DA ⇒ SD) - Histocompatibilty Barrier – Outbred strain				
Group 3: Control DA ⇒ SD	0,0,0,0,0,0,0,0 **(0)**	-	- (no grafts)	100% (8/8)
Group 4: CsA 5 mg/kg	3,3,2,3,3,2,2,3,2,3 **(2.6)**	++	1,1,2,2,2,2,2,1,2,2 **(1.6)**	0 (0/10)
Group 5: MMF 50 mg/kg/d	1,0,1,1,1,2,1 **(1.0)**	(+)	4,4,4,3,4,2,4 **(3.6)**	100% (7/7)
Group 6: CsA & MMF	3,3,4,3,4 **(3.4)**	++	0,1,0,1,0 **(0.4)**	0 (0/5)
Group 7: CsA & MMF	3,2,3,3,3,2,3 **(2.7)**	++	1,3,1,1,1,2,1 **(1.4)**	0 (0/7)
Group 8: W3/25 200 µg/d	2,1,2,1,2,2,2,1 **(1.6)**	+ (+)	2,3,3,3,2,3,3 **(2.6)**	38% (3/8)
Group 9: W3/25 500µg/d	2,3,3,2,2,2 **(2.3)**	++	2,2,1,2,2,2 **(1.8)**	0 (0/6)
Group 10: W3/25 500 µg/d – 30 days	3,2,2,3,2,1 **(2.2)**	++	3,3,3,2,3,3 **(2.8)**	17% (1/6)

Group 11: DST 1x 3 ml.	0,0,0,0,0 **(0)**	-	- (no grafts)	100% (5/5)
Group 12: DST & CsA	0,0,0,0,0 **(0)**	-	- (no grafts)	100% (5/5)
Group 13: DST & W3/25 500 µg/d	2,2,1,1,2,1 **(1.5)**	++	3,2,2,3,2,3 **(2.5)**	50% (3/6)
Group 14: DST & W3/25 & CsA	3,4,3,3,4,3,3 **(3.3)**	++	1,1,1,1,1,1,1 **(1.0)**	0 (0/7)
Group 15: DST & W3/25 & CsA - 30 days	3,3,4,3,3,4,3,3,3 4,3,4 **(3.3)**	+++	2,2,1,2,2,1,1,2,1,1, 2,1 **(1.5)**	0 (0/12)
Allografts DA ⇒ PVG Histocompatibilty barrier – Inbred strain				
Group 16: Control DA ⇒ PVG	0,0,0,1,0 **(0.2)**	-	- (no grafts)	100% (5/5)
Group 17: CsA 5mg/kg/d	2,3,3,2,2,2 **(2.3)**	++	1,2,1,1,2,2 **(1.5)**	0 (0/6)
Group 18: MMF 50 mg/kg/d	1,2,1,1,1 **(1.2)**	+	4,3,3,3,4 **(3.4)**	100% (5/5)
Group 19: CsA & MMF	3,3,2,3,3,3 **(2.8)**	++	0,0,0,1,0,1 **(0.3)**	0 (0/6)
Group 20: CsA & MMF – 30 days	3,3,2,2,3,2 **(2.5)**	++	1,1,2,2,1,2 **(1.5)**	0 (0/6)
Group 21: W3/25 500 µg/d	2,1,2,1,2,1 **(1.5)**	+	2,3,2,3,2,3 **(2.5)**	50% (3/6)
Group 22: W3/25 500 µg/d - 30 days	1,1,2,1,2,1 **(1.3)**	+	3,3,2,3,3,3 **(2.8)**	67% (4/6)
Group 23: DST 1x 3 ml	0,0,0,0,0 **(0)**	-	(no grafts)	100% (5/5)
Group 24: DST & CsA	1,2,2,1,2 **(1.6)**	-	4,4,4,4,3 **(3.8)**	40% (2/5)

Group 25: DST & W3/25	2,1,1,2,1 **(1.4)**	+	3,3,3,4,4 **(3.4)**	60% (3/5)
Group 26: DST + W3/25 & CsA	2,3,3,3,3 **(2.8)**	++	2,1,1,1,2 **(1.4)**	0 (0/5)
Group 27: DST + W3/25 & CsA – 30 days	3,2,3,1,2,2,2,1 **(2.0)**	+	1,3,2,3,3,2,3,2 **(2.4)**	25% (2/8)
Diabetic Groups - STZ Induced diabetes				
Autologous diabetic control group (WAG ⇒WAG)				
Group 32: Autologous control – 30 days	4,3,4,4,4,3 **(3.7)**	+++	0,1,1,1,1,1 **(0.8)**	0 (0/6)
Allogeneic diabetic group (DA ⇒ SD)				
Group 34: DST & W3/25 & CsA – 30 days	3,3,3,3,3,2,3,3,2,3 **(2.8)**	+++	1,1,1,1,1,2,1,1,2,1 **(1.2)**	0 (0/10)

Table 33: Histological scoring of foetal pancreatic grafts according to the method described by Guymer and Mandel 1991. Results shown are the graft scores. A score of 4 denotes intact unaltered grafts while a score of 1 represents severe destruction of the grafts. A score of 0 was awarded if no viable graft remained, the presence of islets (graded – to +++). A graft infiltration score of 0 represents no graft infiltration, 1 indicates slight perigraft infiltrate, a marked graft infiltrate scores 2, score of 3 represents diffuse lymphocytic infiltrate throughout the graft and a score of 4 extensive infiltration) and the percentage of grafts rejected (graft scores of 1and 0).

Chapter 8

DISCUSSION

Normal Controls.

Flow Cytometry - Establishing Normal T-Cell Reference Ranges.

The establishment of reliable, accurate CD2, CD4, CD8 and CD25 normal values is an essential part of this study. The CD4 molecule and the effect of various immunosuppressants on the expression/function of this molecule on the T-Lymphocyte is a central focus in this study.

Two-colour FACS analysis showed that the Sprague-Dawley rat peripheral blood lymphocyte CD4 and CD8 profile differed substantially from that of the PVG rat. The normal SD and PVG reference values established by this study were a $CD2^+CD4^+$ percentage of 51.5% and 72.0% respectively and a $CD2^+CD8^+$ percentages of 48.7% and 24.3% for SD and PVG rats respectively. Values for $CD4^+CD25^+$, a marker for early T-cell activation, and a subset of $CD4^+$ T-cells thought to be regulatory cells, were 5.2% and 7.2% for the SD and PVG rats respectively.

In a comparative study Groen *et al.* (1993) showed that a large variation excists in the $CD2^+CD4^+$ (range 51 to 95%) and $CD2^+CD8^+$ (range 5 to 45%) PBL immunophenotypic percentages between different inbred rat strains[1]. Our PVG results, shown in brackets, CD4% - 80% (72%), CD8% - 20% (24.3%) and the CD4/CD8 ratio was 4 (3.1) compared well with the PBL results described by Groen.

Groen *et al.* (1993) showed that the CD4/CD8 ratio of the rat strains he studied ranged from 9.7 to 1.1 but there appeared to be no correlation between the CD4/CD8 ratio, the inducibility of disease or Th-2/Th-1 ratio [1].

Normal Adult Pancreas Histology of the Rat

Light microscopy of the control DA adult rat pancreata showed the typical pancreas architecture with approximately 2% of the pancreas area consisting of islets of Langerhans (endocrine portion) and the remaining 98% of the pancreas consisting of exocrine tissue (ducts, secretory acini) and stroma containing blood vessels and nerves. In the mature DA rat, islets of Langerhans measured between 50 -100μm in diameter with some bigger coalesced islets of up to 300 μm in diameter dispersed throughout the pancreas. These findings are in agreement with those published by Elayat *et al.* (1995)[2].

The mean islet area of the DA was 82 390 μm of which 54.7% of the total islet area consists of insulin positive β-cells. Glucagon positive α-cells, accounting for 21.5% of the mean total islet area, formed a mantle at the periphery of the islet around the central portion of the islet. Somatostatin and pancreatic polypeptide positive cells appear as single cells among the glucagon cells in the periphery of the islets.

The typical organization of endocrine cells into a β-cell core surrounded by the glucagon, somatostatin and pancreatic polypeptide secreting cells has significant physiological importance for carbohydrate metabolism[3]. The glomerular microcirculation further illustrates the complexity of the islet. The central insulin-secreting portion of the islet receives blood from afferent arterioles which form fine glomerular capillary networks radiating towards the periphery of the islets and the surrounding exocrine tissue[3,4]. Blood, enriched with insulin secreted by the central β-cell core, percolates through the capillaries towards the non-β cells periphery and then drains into an acinar capillary network supplying the peri-islet exocrine tissue[4,5]. At a functional level

the numerous nerve fibres, that terminate in the pericapillary space, further complicate the hormonal regulation in the islet of Langerhans[4]. At the cellular level, gap junctions between the endocrine cells also regulate secretion by permitting transfer of low molecular weight substances between adjacent cells[3]. It is therefore clear from the islet structure that hormonal control of insulin, in particular in the islet of Langerhans and the surrounding exocrine tissue, is both complex and multifactorial [3].

Histological Evaluation of Control and Transplanted DA Foetal Pancreatic Tissue 16-18 Days Gestation (Autografts).

Histology of the foetal rat pancreas at 17 days gestation showed undifferentiated tissue consisting of ductular structures and capillaries. Endocrine cells were mostly associated with the ducts and had not yet formed morphologically recognizable islets of Langerhans. Secretory acini were absent. Brown *et al.* (1982) proposed that, due to the absence of developed exocrine tissue, this was the preferred gestation age for FRPT, yielding the highest proportion of endocrine tissue following engraftment [6].

Immunocytochemistry showed that very few differentiated endocrine cells were present at this stage and that the insulin and glucagon positive cells were present either as single cells or as small clusters associated with the ductules. Somatostatin positive cells were rare. These findings concur with the chronological appearance of endocrine cells in the developing rat, as described by McEvoy *et al.* in 1980. He showed the appearance of glucagon cells at 12 days gestation followed by insulin cells at 15 days and somatostatin on gestational days 17 – 18 [7,8].

Autologous engraftment of 16 – 18 day gestation FRP allows for the continued development of the endocrine component of the pancreas. By 3 days post-transplantation the insulin positive

cells have formed small clusters of cells closely associated with the ductules. At this stage many mitotic figures of ductular cells are seen. At 5 days post-transplantation, the insulin positive clusters/cells have increased in size and, although still associated with the ductules, they start to take on the morphological appearance of islets of Langerhans. By 10 days post-transplantation, most of the islets are no longer associated with the ductules and appear loose in the stroma. At this stage the ductules start showing signs of atrophy with widening of their lumens. The islets continue to grow and, by days 20 and 30 post-transplantation, they take on the appearance of mature islets, which consist almost entirely of insulin positive cells. At this stage some remnant ductules, characterized by their wide lumens, are still present in the grafts. The appearance of peri-graft subcapsular fat during this period, possibly related to the ectopic secretion of insulin by the islets, is a common observation [9].

In contrast to insulin positive endocrine cell growth, the glucagon positive endocrine cells, which are the first to appear in the normal developing pancreas, appeared to be present in reduced amounts per islet [7,8,10,11]. By 10 days post-transplantation the occasional glucagon positive cell is seen at the periphery of the developing islet and, although there is a slight increase in the number of glucagon positive cells at days 20 and 30, it is still far less than seen in the normal adult pancreatic islet. The possible cause and effect of the decreased α-cell (glucagon) presence in the transplanted islets is not known. Factors such as denervation, ischaemia or exocrine growth factors may play a role but there is no literature to support these views.

Somatostatin positive cells are present in the transplanted islet, appearing as single cells in the periphery of the developing islet and detectable from 10 days post-transplantation by immunocytochemistry.

NON-IMMUNOSUPPRESSED TRANSPLANT ALLOGRAFT CONTROLS.

Flow cytometry, performed pre- and post-transplantation, showed that the procedure did not have a significant effect on either the $CD2^+CD4^+$, $CD2^+CD8^+$ or $CD4^+CD25^+$ PBL immunophenotypes.

Histology of the harvested non-immunosuppressed transplantation control grafts showed fierce rejection of the DA ($RT1^{av1}$) donor strain pancreata in both the outbred SD and the inbred PVG ($RT1^c$) recipients. Although the DA ($RT1^{av1}$) to PVG ($RT1^c$) strain is described as a low responder the strain rejected the FRPT completely by 14 days post-transplantation[12,13]. In all cases, the transplanted renal subcapsular foetal pancreata were completely rejected and replaced by fibrous tissue within 14 days of transplantation. In most cases a mononuclear cell infiltrate was still present mainly at the kidney graft interface.

CSA, MMF AND CSA/MMF: COMBINATION IMMUNOSUPPRESSION IN TRANSPLANTATION ALLOGRAFT GROUPS.

Flow cytometric monitoring of the peripheral blood T-lymphocytes in the SD rat, following FRP transplantation and daily CsA monotherapy (5 mg/kg/d), showed a significant decline in PBL $CD2^+$, $CD2^+CD4^+$, $CD2^+CD8^+$ and the CD4/CD8 ratio 1 day post-transplantation. Although the $CD2^+$ and $CD2^+CD8^+$ and the CD4/CD8 ratio levels had recovered by days 7 and 14 post-transplantation the mean $CD2^+CD4^+$ percentages were still marginally lower than the pre-transplantation levels. CsA monotherapy had a similar effect, following FRP transplantation, in the PVG rat. The PBL $CD2^+CD4^+$ immunophenotype showed a significant decline on day 7

post-transplantation, resulting in a reduced CD4/CD8 ratio. The $CD2^+CD4^+$ and the $CD4^+CD8^+$ levels recovered to non-significant levels by day 14 post-transplantation. The short term transient $CD2^+CD4^+$ PBL lymphocytopenia, following FRPT and CsA treatment, has also been reported following surgical trauma in humans: the observations of our study and could therefore be due to the animals recovering from the operation and acclimatising to the CsA therapy [14].

Mycophenolate mofetil monotherapy at the given dose (50 mg/kg/d) resulted in a slight (5%) decline in $CD2^+CD4^+$and a marginal increase of 13% in the $CD2^+CD8^+$ percentage of the PVG rats (p=NS) but had almost no effect on PBL imunophenotype of the SD rats. It would appear as if MMF does not have an immediate measurable immunosuppressive effect on the PBL and that it is well tolerated by the rat.

Cyclosporine and MMF combination therapy, at the given dose, resulted in significant decline of PBL $CD2^+CD4^+$ percentage of both the SD and PVG experimental animals by days 14 and 30 post-transplantation. The $CD2^+CD4^+$ PBL decline resulted in a significant increase of the $CD2^+CD8^+$ percentage and therefore a significant decline in the CD4/CD8 ratio.

Cyclosporine suppresses activation transcription factor genes that are necessary for cytokine production (including IL2, IFN-γ, GM-CSF, TNF-α and IL-4). It therefore inhibits downstream lymphocyte proliferation, resulting in a general suppressive effect on activated PBL T-cells, B-cells and monocytes. MMF, known to be synergistic with CsA, prevents *de novo* purine synthesis and, because activated lymphocytes lack the purine salvage pathway, MMF effectively prevents clonal expansion[15-18]. Why CsA and MMF combination therapy affected $CD2^+CD4^+$ PBL's, more than $CD2^+CD8^+$, is open to speculation but probably relates to the activation status and T-cell subset regeneration of the lymphocytes during the period. Persistent CD4+

lymphocytopenia, disproportionate $CD2^+CD8^+$ regeneration and a consequent decline of the CD4/CD8 ratio have been described in MMF/CsA treated human renal recipients and in mice[19,20,21]. The $CD4^+CD25^+$ PBL percentages were unaffected by CsA and MMF either as monotherapy or in the combination therapy groups (p=NS).

The histology confirmed that, at the given dose (5 mg/kg/d), CSA consistently prevented acute rejection in both the SD and PVG transplanted animals, allowing for the development, predominantly, of the endocrine component (islets of Langerhans) following FRPT. Graft/infiltrate scores, in these groups, reflected good graft survival with light to moderate mononuclear cell infiltrates at the time of harvest – 14 days post-transplantation. These results concur with the findings of Brown *et al.* (1988) who demonstrated the efficacy of CsA monotherapy, at 15 mg/kg/d, in preserving the graft and limiting the mononuclear infiltration into foetal pancreas allografts in the DA ($RT1^a$) to PVG ($RT1^c$)[22].

In contrast to CsA, MMF monotherapy, at the given dose (50 mg/kg/d), failed to prevent rejection at 14 days and showed only remnants of the rejected graft, amongst a massive mononuclear cell graft infiltrate, in both the SD and PVG groups. These findings are similar to those achieved by Schulak *et al.* (1996) who showed that MMF was ineffective as a monotherapy in preventing rat pancreas allograft rejection [23].

Combination of CSA and MMF (at the given dose of 5 mg/kg/d and 50 mg/kg/d respectively) resulted in excellent graft survival and development of the grafts in both the SD and PVG recipients which were essentially infiltrate free at the time of harvest 14 and 30 day post-transplantation. Excellent graft scores, with low infiltrate scores, were achieved in the SD

recipients at 14 and 30 days post-transplantation, and this was marginally better than what was achieved in the PVG recipients.

The low graft infiltrate scores, associated with CsA and MMF combination therapy, could be of clinical importance in the prevention of chronic rejection which, despite the introduction of new immunosuppressants, is still a major cause of graft loss [24].

Electron microscopy of the islets, 30 days post-transplantation, confirmed the presence of viable and functional endocrine cells containing numerous secretory granules, most of which had the characteristic dense core insulin-like appearance. The presence of numerous capillaries, between the endocrine cells, indicated that the islets were well vascularised and therefore viable.

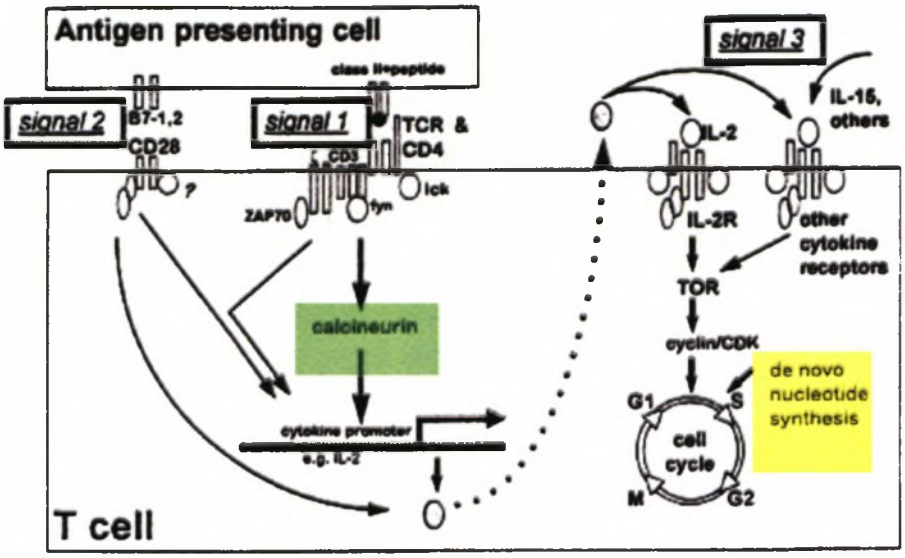

Figure 108: An illustration demonstrating the highly effective two-stringed bow approach to immunosuppression when using CsA and MMF therapy. CsA, by inhibiting calcineurin (highlighted in green), acts early in the lymphocyte activation pathway, preventing the transcription of various cytokine genes (eg. IL-2), which are necessary for the activation signal 3 in the illustration above. MMF acts late in the activation pathway by inhibiting the enzyme IMPDH. It effectively inhibits purine synthesis (highlighted in yellow) and therefore lymphocyte proliferation[15-18] (Illustration by Halloran in Am. J. Med. Sciences 1997; 313(5): 283 – 288).

ANTI-CD4 (W3/25) MONOCLONAL ANTIBODY IMMUNESUPPRESSION OF TRANSPLANTATION ALLOGRAFT GROUPS.

Flow cytometric analysis showed that a single intravenous dose of 500 µg W3/25 resulted in a rapid 33.2% decrease of the $CD2^+CD4^+$ PBL percentage in the adult 450 g SD rat, within 10 minutes of the antibody infusion. Subsequently, the $CD2^+CD8^+$ PBL percentage increased by 38% and the CD4/CD8 ratio declined from 1.5 to 0.7. In 1995, Pelegí et al. hypothesised that the reduction of $CD4^+$ cells from the peripheral blood circulation, following W3/25 in vivo

administration, was due to a decrease in CD4 antigen expression or a reduction of circulating $CD4^+$ lymphocytes [25].

In this study daily administration of 200 μg W3/25, to the SD recipients, caused the $CD2^+CD4^+$ PBL percentage to decline significantly by 42.6% from pre-transplantation values to 14 days post-transplantation (p=0.008). The $CD2^+CD8^+$ PBL percentage increased significantly in this period, by 49.2%, resulting in a significant decline in the CD4/CD8 ratio to 0.4. Of interest is that the $CD2^+$% of the gated lymphocytes declined by 20% in the 14-day period but this was not significant.

Increasing the daily dose of W3/25 from 200 to 500 μg further reduced the $CD2^+CD4^+$ PBL percentage by 65.7% at 14 days and by 66.7% at day 30 post-transplantation. The $CD2^+CD8^+$ PBL percentage increased by 72.6% and 62.4% at 14 and 30 days post-transplantation. Subsequently the CD4/CD8 ratio was reduced to 0.2 by 30 days post-transplantation. The non-parametric Spearman Rank test, to test for a correlation between the $CD2^+CD4^+$ decrease and the $CD2^+CD8^+$ PBL percentage increase, gave a significant negative correlation. An important feature of the W3/25 treatment is the gradual decline of $CD2^+$ percentage from the lymphocyte gate (FSC vs. SSC) reaching significance by day 30 (from 34.7% to 20.9%: p=0.03). Taking the above into account it becomes clear that following W3/25 administration, the $CD2^+CD4^+$ PBL's are rapidly removed or migrate from the peripheral circulation. This is supported by the significant correlation between the declining $CD2^+CD4^+$ percentage and the increasing $CD2^+CD8^+$ PBL percentage, and the declining $CD2^+$ percentage from the lymphocyte gate, rather than the down regulation of surface CD4 expression of the $CD2^+CD4^+$ PBL's, as suggested by Pelegí *et al.* [25]. The significant decrease in circulating host $CD4^+$ cells following W3/25 administration has been described by Sablinski *et al.* (1991)[26].

An interesting observation was the significant increase of $CD4^+CD25^+$ PBL's following W3/25 administration at 14 and 30 days post-transplantation. CD25 is a marker both for early activation of CD4 T-cells and for a subset of CD4 T-cells, which include regulatory T-cells. It is difficult to speculate if the increase in the $CD4^+CD25^+$ PBL percentage is induced by the W3/25 therapy or whether, in fact, these are either established $CD4^+CD25^+$memory/suppressor cells, which are not migrating from the peripheral circulation, or more likely are newly released $CD2^+CD4^+$, or even $CD4^+CD8^+$, cells from the thymus which have become activated [27].

In the PVG, 500 μg W3/25 had a similar effect on the $CD2^+CD4^+$ subset, resulting in the significant decline of $CD2^+CD4^+$ by 40.6% and 60.3% and a $CD2^+CD8^+$ percentage increase of 105.7% and 206.6% at 14 and 30 days post-transplantation respectively. The non-parametric Spearman Rank test showed a negative correlation between the $CD2^+CD4^+$ and $CD2^+CD8^+$ percentage. In clinical transplantation the use of ATG results in long-term, (up to 5 years after transplantation) depletion of $CD4^+$ cells and an increased regeneration of $CD8^+$ cells, especially the $CD8^+CD57^+$ subpopulation[21].

In the PVG the $CD2^+$%, within the lymphocyte gate, declined significantly following W3/25 treatment, by 55.3% and 61.6% from the pre-transplant levels at 14 and 30 days post-transplantation, indicating that W3/25 treatment had the same effect on the SD and the PVG $CD4^+$ and $CD8^+$ T-lymphocyte subsets. Following W3/25 treatment, the $CD4^+CD25^+$ PBL subset in the PVG was slightly increased but, in contrast to the SD, failed to reach significance.

Following 200 μg W3/25 per day, histology of the grafts showed slight graft survival but with a marked mononuclear cell infiltrate consisting mostly of small lymphocytes. In an attempt to improve mean graft/infiltrate score, the dose of W3/25 was increased from 200 μg/d to 500 μg/d.

The higher dose improved the graft score but failed to maintain a low infiltrate score at 30 days post-transplantation. The graft survival and development of islets of Langerhans in the SD rat was comparable to that achieved with CsA monotherapy but with a slightly higher graft infiltrate score. Grafts harvested from the PVG rats, at 14 - and 30 days post-transplantation, showed low graft survival but the graft/infiltrate scores were not markedly improved. Although the PVG scores following W3/25 treatment are disappointing, variation in the efficacy of anti-CD4 treatment in different strain combinations is a well-described phenomenon. In some strains the activation of the rejection response is totally dependent on $CD4^+$ cells[28]. In these strains, the hosts develop specific tolerance to the graft however, in strain combinations in which $CD8^+$ cells can be activated without $CD4^+$ help, anti-CD4 therapy simply results in a delayed rejection of the grafts [28]. In *in vitro* MLC results, the DA ($RT1^{avl}$) and PVG ($RT1^c$) strain combination confirmed the presence of a population of CD4+ T-cells, which were refractory to the inhibitory action of W3/25 on T-cell activation[29]. Qi *et al.* (1997) identified the DA ($RT1^a$) strain as a low responder characterized by the inability of its pure CD8 population to proliferate or reconstitute rejection following antigen presentation[30]. Tolerance was easily achieved, by a single dose of a depleting anti-CD4 (OX-38), in the PVG ($RT1^c$) to DA ($RT1^a$) low-responder strain combination. By switching donor and recipient strains, tolerance could not be achieved in the DA ($RT1^a$) to PVG ($RT1^c$) constituting a high-responder strain combination[30]. This could explain why W3/25 therapy was less effective in our studies in the PVG than in the SD rat.

DONOR SPECIFIC TRANSFUSION (DST), CYCLOSPORINE (CSA) AND ANTI-CD4 (W3/25) INDUCTION THERAPY GROUPS – DONOR SPECIFIC ANTIGEN INDUCED UNRESPONSIVENESS.

Medawar first demonstrated the potential benefit of donor specific transfusion to the recipient, prior to transplantation, in 1946. In clinical transplantation, the use of DST remains controversial, but is still used, in combination with conventional immunosuppression, by some transplantation units [31].

Donor specific transfusion, in combination with CsA induction therapy had no significant effect on the T-cell immunophenotypes, apart from a drop in $CD2^+CD4^+$ PBL cell percentage 1-day post-transplantation. Combining DST with CsA induction therapy (x5 days) resulted in accelerated rejection of the grafts in the SD rat. However in the PVG, the response was less acute and, although there was a pronounced lymphocytic infiltrate present, some graft elements were still identifiable.

Woods, a renowned transplantation immunologist at Oxford, first demonstrated that specific donor tolerance could be achieved in the mouse and rat, if DST was given in combination with anti-CD4 28 days prior to transplantation[32,33,34].

In this study, the potential of DST was utilized as an adjunct to anti-CD4 and CsA, specifically to induce graft unresponsiveness in the strong responding DA to SD and PVG, FRPT model.

Flow cytometry analysis, following a single 3 ml DST, showed a slight, but not significant decline of 5.9% and 1.4% in the $CD2^+CD4^+$ population and a 10.5% and 26.1% disproportionate increase in $CD2^+CD8^+$ of the SD and PVG respectively, at 14 days post-transplantation. The $CD4^+CD25^+$ expression also increased slightly in the SD rats but had declined slightly in the

PVG group 14 days post-transplantation. Although these changes were not significant and were within established normal ranges.

Professor Bert Myburg, an eminent transplantation biologist, at the Witwatersrand Medical School suggested (oral communication) that the inadvertent introduction of viruses into SPF rats, used as transplantation models, often plays a major role in the outcome of these experiments and this is seldom appreciated by researchers in the field. For example, an increase in $CD8^+CD57^+$ cells has been described in patients with CMV infection[19]. An increase in the $CD4^+CD8^+$ cells, which normally represent approximately 6% of the T-cells in the peripheral blood of the adult rat, could also account for a disproportionate increase in $CD2^+CD8^+$ [27].

Flow cytometry of the DST and CsA combination therapy groups showed no significant changes in the PBL immunophenotype combinations, at 14 days post transplantation, except for a slight, but not significant, increase in the $CD4^+CD25^+$ expression in the PVG group. On day 1 post-transplantation in the SD group, the significant changes in the values of all the T-cell subsets did not fit in with the general trend: this can only be due to technical problems either with the staining or the FACS analysis on the specific day. In comparison to the CsA monotherapy groups, the DST and CsA combination resulted in similar non-significant changes in the T-cell immunophenotypes.

Combining DST and W3/25 resulted in a significant (51.7%) decrease in the $CD2^+CD4^+$ percentage in the SD rat while, in PVG rats, a more moderate but still significant 17.3% reduction could be demonstrated one day after commencement of treatment. Although W3/35 administration was intentionally stopped at 5 days, $CD2^+CD4^+$ percentages remained significantly lower in both SD and PVG rats 14 days post-transplantation. In response to the

W3/25 induced $CD2^+CD4^+$ PBL lymphocytopenia, $CD2^+CD8^+$ percentages increased by 47.1% and 58.3%, 14 days post-transplantation in SD and the PVG rats respectively. DST and W3/25 combination therapy resulted in a significant 28.7% decline of FSC vs. SSC gated CD2+ cells in the SD rats. In the PVG rats, the CD2 cells were still 15.2% lower at 14 days post-transplantation but this was not significant. Apart from a non-significant increase in the SD rat on day 14, DST and W3/25 induction therapy had no significant effect on the $CD4^+CD25^+$ percentage. In comparison to the 500µg/d W3/25 monotherapy groups, the $CD2^+CD4^+$ suppression, induced by DST and W3/25, was less marked, and although still significantly lower at day 14 post-transplantation, showed some signs of recovery following the withdrawal of W3/25 therapy on day 5.

Using 3 mg W3/25 doses, administered on days 0, 2, 4, 7, 9, 11 and 14 to an adjuvant arthritis Wistar rat model, Pelegri *et al.* (1995) found that all $CD4^+$ cells were removed from the circulation by day 8, after 4 doses. $CD4^+$ cells were only detected on day 17, 3 days after the last dose of W3/25 was given, and only returned to normal levels on day 49. $CD8^+$ cells were found to be significantly higher on days 24, 38 and 49[25]. The findings of Pelegri *et al.* (1995) corresponded with the $CD4^+$ decline and the $CD8^+$ increase, following 5 days of W3/25 administration in our model. However Pelegri *et al.* (1995) hypothesizes that the decline of peripheral blood $CD4^+$ cells was due to a W3/25 induced down-regulation of CD4. They based these findings on single-colour indirect immunofluorescence FACS analysis which showed the absence of a positive peak but a wider than normal negative peak. By using two-colour directly conjugated FACS analysis, I believe this study shows that the $CD2^+CD4^+$ cells are eliminated or migrate from the peripheral circulation. The decline of $CD2^+$ cells from the lymphocyte gate would support this hypothesis. Two-colour FACS showed no sign of a $CD4^{dim}$ population, as

this would correspond with the wider negative peak described by Pelegri *et al.*[25]. The secondary antibody used by Pelegri *et al.* in the indirect immunofluorescence method, was a FITC conjugated goat anti-mouse IgG which would detect any exogenous W3/25 and result in such a broader negative peak. Using *in vitro* blast cultures, Stumbles *et al.* (1995) found that W3/25 uniformly bound to all $CD4^+CD5^+$ T-cells without modulating the cell surface expression of CD4 antigen[35].

The rapid reduction of circulating $CD2^+CD4^+$ cells from the peripheral blood, although recovering slightly after stopping therapy, evokes the issue of the relevance of depleting and non-depleting antibodies. W3/25, a mouse anti-rat CD4 mAb of IgG_1 isotype, is described as a non-depleting antibody[35]. This is due to its inability to elicit antibody-dependent cellular cytotoxicity and complement-dependent cytolysis[35]. *In vivo*, our results suggest that W3/25, by removing the CD4 T-cells subset, acts more like a depleting antibody in the rat strains we utilized[35,36].

The effect on the T-cell immunophenotypes was greatly enhanced in the DST, CsA and W3/25 combination group, with significant declines of $CD2^+$, $CD2^+CD4^+$ and the CD4/CD8 ratio of the SD rat at 30 days post-transplantation i.e. 25 days after CsA and W3/25 therapy was stopped. The $CD2^+CD8^+$ PBL percentage was still greatly increased by 41.7% from pre –treatment levels and the $CD2^+CD25^+$ percentage was also higher at 30 days post-transplantation, (p= NS). In the PVG rat the combination therapy had a shorter lasting effect. At 7 days post-transplantation i.e. 2 days after termination of treatment, the $CD2^+$, $CD2^+CD4^+$ and CD4/CD8 ratio was significantly lower while the $CD2^+CD8^+$ was significantly higher that pre-treatment values. By 30 days post-transplantation, apart from the $CD2^+$ T-cells, all phenotype values had recovered to near normal but these values did not differ significantly from controls.

Although the W3/25 effect of eliminating circulating $CD2^{+}CD4^{+}$ T-cells from the peripheral circulation is similar in both the SD and PVG rat, it is clear from the flow cytometry results, following W3/25 treatment, that the immunological effect in the SD is more severe and longer-lasting following W3/25 monotherapy, DST and W3/25 therapy or DST, CsA and W3/25 combination therapy. In a review article by Sablinski *et al.* (1991) the divergent mAb effects *in vivo*, were alluded to and may be due to the diversity of low- and high-responder animal strains[26].

Histology

A single 3 ml dose of DST did not prevent the acute rejection of the FRPT in either the DA to SD or DA to PVG rat model. This finding was not unexpected. By 14 days post-transplantation all the grafts in the DST monotherapy groups were completely rejected and replaced by fibrous tissue with some focal areas of mononuclear cell infiltrates. DST, as a monotherapy, is generally not effective in preventing allograft rejection and generally requires some adjunct immunosuppression to achieve graft acceptance. This has also been documented in clinical transplantation in man. However Kataoka *et al.* (2002)[37] demonstrated indefinite survival of more than 100 days of Lewis (RT1l) cardiac allografts into DA (RT1a) - low responder model. This finding was confirmed by Hamano *et al.* (1989)[38]. If our DA to SD and PVG strain combinations, are compared to the non-immunosuppressed controls, DST monotherapy had no effect or FRP graft survival.

Grafts harvested 14 days after transplantation, following DST and CsA (5 days) induction therapy, displayed accelerated rejection in the SD rat strain. The grafts were completely rejected and consisted of fibrinoid material and mononuclear infiltrates, typical of immunological destruction. Rejection was not as fierce in the PVG rats, with some ducts and islets of

Langerhans still surviving within a pronounced mononuclear cell infiltrate. Tchervenkov *et al.* (1995) showed a synergistic effect between DST and CsA in the Lewis to DA model. This synergy was dose dependent, high CsA doses were detrimental while the length of CsA coverage contributed to graft survival[39]. In our groups, CsA administration for 5 days was probably insufficient to establish graft unresponsiveness in our high responder models. Frede *et al.* (1997) showed that combining DST with a 14 day course of CsA, significantly prolonged cardiac allograft survival in the ACI ($RT1^a$) to Lewis ($RT1^l$) strong responding allograft model. These workers showed that DST and CsA therapy caused an early shift to a Th-2 phenotype but that additional immunosuppression was required to establish long-term unresponsiveness and to combat rejection episodes[40].

Histology of the harvested grafts, following DST and W3/25 induction treatment, yielded disappointing results in both the SD and PVG groups, from a rejection point of view. In both cases, minimal viable graft elements, including islet components, could be identified amongst a pronounced mononuclear cell infiltrate. The use of DST and anti-CD4 mAb is a well-established protocol for inducing donor specific tolerance in certain rodent strains. In rodents it takes 28 days to establish the immunological unresponsiveness by regulatory T-cells, which forms part of the $CD4^+CD25^+$ subset[41,42]. The heavy infiltrate into the graft, following DST and W3/25 combination induction therapy, suggests that additional immunosuppression is needed to reduce graft rejection, while allowing the establishment of donor specific T-cell regulation. To achieve this, CsA was therefore included together with the DST and W3/25 induction therapy.

DST, CsA and W3/25 combination treatment in the SD rat yielded excellent well-preserved grafts at 14 and 30 days post-transplantation, with almost no graft infiltration. Histology of the grafts, 30 days post-transplantation, showed the presence of well developed and near normal

islets of Langerhans within a loose fibrous stroma. The islets were histologically shown to consist chiefly of insulin and a few glucagon positive cells indicating that the islets were functioning from an insulin point of view. The reason for the reduced glucagon component remains speculative. Renal subcapsular adipose tissue associated with the grafts, possibly due to ectopic insulin secretion, was a common observation and has been reported, previously [9].

In the PVG rat the DST, CsA and W3/25 combination induction therapy resulted in excellent histological graft survival with a mild to moderate graft infiltrate at 14- and 30 days post-transplantation. Immunofluorescence labelling showed that the graft infiltrate consisted of both $CD4^+$ and $CD8^+$ cells. The lack of rejection in the presence of the infiltrate could indicate active graft specific suppression by suppressor or anergic T-cells or might be due to chonic rejection of the graft.

Immunocytochemistry confirmed that the islets consisted mainly of insulin positive endocrine cells, which is a positive outcome in a strong responding allograft model.

Although the SD rats responded better to the DST, CsA and W3/25 induction therapy than the PVG rats, this protocol effectively prevented rejection of the FRPT in both rat strains without the need for continuous daily immunosuppression. This observation is of great immunological importance and has direct clinical application.

DIABETIC GROUPS – REVERSAL OF STREPTOZOTOCIN (STZ) INDUCED DIABETES BY AUTOLOGOUS AND ALLOGENEIC FOETAL RAT PANCREATIC TRANSPLANTATION (FRPT).

The diabetic model.

Streptozotocin, a glucosamine derivate with well know beta-cytotoxic properties, depletes insulin-producing β-cells in a dose dependent manner[43,44]. In the diabetic model a dose must be selected that does not result in uncontrollable ketotic diabetes, which is considered to be detrimental to the development of islets of Langerhans following FRPT[45].

Injection of 50 mg/kg STZ, in age-matched and genetically identical rats, resulted in unpredictable levels of hyperglycaemia, necessitating selection of diabetic animals with non-fasting WBG values of between 10 and 20 mmol/l [45]. Although several groups used this model as a model for type I diabetes, the difficulty of using STZ to obtain a modest and reproducible elevation of glucose levels has been acknowledged previously[46].

Isogeneic (autologous) Foetal Rat Transplantation of STZ induced Diabetic Rats.

Syngeneic renal subcapsular foetal pancreas transplantation has the potential to normalize the gross clinical and metabolic disorders of streptozocin-induced diabetes mellitus in a rat model and maintaining near normal blood glucose levels [47-51]. Normoglycaemia was consistently restored 30 days post-transplantation, by autologous foetal pancreas transplantation, resulting in non-fasting whole blood glucose levels in the range of normal controls. Although the rats gained weight and appeared to grow normally after transplantation, they had not reached the weight of the normal age-matched controls, probably due to STZ toxicity and the metabolic deficiencies of experimental diabetes. The metabolic profile showed that the clinical signs of insulin-dependent

diabetes in the rat model i.e. polydipsia, polyuria, glucosuria and wasting had been consistently and permanently reversed. These findings are similar to those documented after isogeneic isolated renal subcapsular islet transplantation[52,53].

Intravenous glucose tolerance tests showed that the transplanted animals were able to mount a response to the glucose bolus, returning to baseline glucose levels at 40 minutes. This compared well with controls which returned to basal values at 30 minutes. Comparison of the control glucose clearance rate, of the control and isograft animals showed that the K-values had been restored to 85% of normal at 30 days post-transplantation. Analysing the area under the IVGTT-curve showed that the glucose metabolism had been restored to 88% of normal. Although both the K-values and the area under the IVGTT-curve showed a subnormal response to the glucose bolus, one must acknowledge that the IVGTT is far beyond the normal dietary carbohydrate load and therefore probably reflects on the reserve capacity rather than the physiological requirement[53]. It is but one instrument to test glycaemic control. In humans the oral glucose tolerance test is preferred to IVGGT as the insulin response is more physiological.

Histologically, the grafts showed a classic development, with the exocrine tissue being replaced with fibrous tissue at 30 days post-transplantation. The islets were well developed within the fibrous stroma or in the perigraft white adipose tissue. Immunocytochemistry confirmed the near normal endocrine morphology of the islets, with the majority of the cells within the islets staining positive for insulin. This has been reported previously and this work supports the findings of other researchers[54,55].

Morphological analysis of the different islet cell populations showed that β-cell volume did not differ significantly from controls, but that a significant decrease of α-cells as measured by % α-

cell area/islet area and α-cell size was present. The suppression of α-cell development in the grafted islets could be due to some form of negative feedback from functional α-cells present in the pancreas. The outcome of poorer distribution of glucagon positive staining α-cells and other components within the transplanted islets is however not fully understood. However, isografts have a near normal complement of insulin producing cells that are capable of restoring normoglycaemia in diabetic rats. These studies show that this outcome can be consistently achieved and that the results are durable as no animals reverted back to the diabetic state for the duration of the study. Revascularisation and reinnervation of the grafts are crucial for the function and survival of the islets[56]. Although we were unable to demonstrate reinnervation of the islet using neurofilament protein, neuron-specific enolase and S100 protein, Adeghate (2002) demonstrated that early reinnervation of islets transplanted into the anterior rat chamber of the eye had occurred within 5 days of transplantation[56,57]. Adeghate demonstrated the presence of extrinsic nerves containing calcitonin gene-related peptide, galanin, neuropeptide Y and choline-acetyl-transferase within the 5-day transplanted islets[56].

Allogeneic Foetal Rat Transplantation of STZ induced Diabetic Rats.

Streptozotocin induced diabetes, as shown in this study and by others, can be successfully reversed by isogeneic FRPT. It should therefore theoretically be possible to reverse STZ induced diabetes in the rat by allogeneic transplantation provided rejection could be combated. In practice, however, rejection and the significant diabetogenic side effects and obstacles associated with steroid and calcineurin inhibiting immunosuppressants have proved difficult to overcome. By combining DST with a short course of anti-CD4 (W3/25) and CsA, graft unresponsiveness was achieved without the need for daily immunosuppression. The

immunemodulation induced by this treatment protocol resulted in sufficient graft growth and maturation to reverse the clinical signs of the streptozocin induced diabetes mellitus in the DA to SD rat model and to sustain normoglycaemia. Following allogeneic FRPT, non-fasting euglycaemic blood glucose levels were constantly achieved and maintained for 30 days post-transplantation. Following FRPT, metabolic aberrations including weigh-loss, polydipsia, polyuria, glucosuria were functionally reversed.

Intravenous glucose tolerance tests showed that in contrast to the diabetic controls, the allogeneic transplanted recipients where able to respond to the glucose bolus although, as with some normal controls, terminal blood glucose levels were still slightly elevated at 60 minutes. The area under the IVGTT-curve showed that the AUC glucose metabolism had been restored to 80% of normal and the control glucose clearance rate showed the K-values were restored to 85% of normal values at 30 days post-transplantation.

Macroscopically, the grafts appeared well vascularised and associated with subcapsular white adipose tissue as seen in the isogeneic model. Histologically, the grafts consisted of well-developed islets of Langerhans and atrophying exocrine tissue, mostly dilated ducts. Very few graft infiltrates were present within the grafts. Immunocytochemistry showed that the islets consisted mostly of insulin positive endocrine cells. Electron microscopy confirmed the ultrastructural viability of the endocrine cells.

Morphological analysis showed that, despite the ability of allogeneic foetal islets to reverse the STZ-induced diabetes, these islets were significantly smaller and contained fewer β-cells per islet area than pancreatic islets of the normal controls. The β-cell/α-cell ratio of the transplanted area

was however 5 times higher than that of the normal pancreatic islets. These findings are similar to those described by McEvoy *et al.* (1978)[58].

The extent to which diabetes was reversed, following allogeneic transplantation, was comparable to that achieved by isogeneic transplantation. The genetic variation between the two rat strains used, and the biological variation of the STZ diabetes model, makes it impossible to make a meaningful quantitative comparison between the diabetic isogeneic transplantation and the allogeneic transplantation groups. From the results of the isogeneic and the allogeneic transplantations groups and controls, it is clear that allogeneic foetal rat pancreatic transplantation is a treatment modality capable of restoring normoglycaemia in the diabetic rat laboratory model and therefore has great potential for clinical relevance and application.

BIBLIOGRAPHY

[1] Groen H, Van Petersen FA, Pater AS, Nieuwenhuis P, Kampinga J. Composision of $CD4^+$ resting memory T-cell pool is influenced by the major histocompatibilty complex. Transplant Proc 1993; 25: 2782–2783.

[2] Elayat AA, El-Naggar MM, Tahir M. An immunocytochemical and morphometric study of the rat pancreatic islets. J Anat 1995; 186: 629-637.

[3] De Groot LJ, Besser M, Jameson JL, Loriaux DL, Marshall JC, Odell WD, Potts JT, Rubenstein AH. Endocrinology volume 2, 3rd edition; Philadelphia: W.B. Saunders Company 1993.

[4] Zhou Z-G, GAO X-H, Wayand W-U, Xiao L-J, Du Y. Pancreatic microcirculation in the monkey with special reference to the blood drainage system of Langerhans islets: light and electron microscopic study. Clinical Anatomy 1996; 9: 1–9.

[5] Henderson JR, Daniel PM. The pancreas as a single organ: the influence of the endocrine upon the exocrine part of the gland. Gut 1981; 22: 158–167.

[6] Brown J, Heininger D, Kuret J, Mullen Y. Normal Response to Pregnancy in Rats Cured of Streptozotocin Diabetes by Transplantation of One Fetal Pancreas. Diabetologia 1982; 22: 273-275.

[7] McEvoy RC, Madson KL. Pancreatic insulin -, glucagon-, and somatostatin-positve islet cell populations during the perinatal development of the rat. I Biol Neonate 1980; 38: 248-254.

[8] Yoshinari M, Daikoku S. Ontogenic appearance of immunoreactive cells in rat pancreatic islets. Anat Embryol 1982; 165: 63–70.

[9] Du Toit DF, Mouton YM, Muller CJF. The morphology of peri- and intragraft white adipose tissue (WAT) ingrowth following foetal rat pancreatic transplantation. European Journal of Anatomy 1997; 1 (3): 129–136.

[10] Du Toit DF, Muller CJF, Page BJ, Louw J. Foetal rat pancreatic transplantation: posttransplantation development of foetal pancreatic iso- and allografts and suppression of rejection with mycophenolate mofetil (MMF) and cyclosporine based immunesuppression. Microscopy Research and Technique 1998, 43 (8), 347–355.

[11] Muller CJF, Du Toit DF, Page BJ, Louw J, Muller BJF, Williams K, Mattysen JJ, Lyners RFC. Islet Morphology and Function Following Foetal Rat Pancreatic Transplantation. Eur J Anat 2000; 4(3): 149-160.

[12]Kobayashi E, Lord R, Green M. Detection of membrane-bound and soluble-form MHC class I antigen from rat pancreas/spleen grafts during ongoing rejection. Transplant Proc 1999; 31: 3409–3413.

[13] Yasunaru Sakuma, Hiroo Uchida, Hideo Nagai and Eiji Kobayashi. High-dose tacrolimus and lengthy survival of the combined rat pancreas/spleen graft in a high-responder combination. Transplant Immunology 2001; 9(1): 37-42.

[14] Wramner L, Mjörnstedt L, Blohme I, Tufveson G, Söderstrom T, Olausson M. Alterations in the post transplant blood lymphocyte phenotype subsets as a marker of rejection in renal allograft recipients. Scand J Immunol 1995; 42: 275–281.

[15] Halloran PF. Immunosuppressive agents in clinical trials in transplantation. Am J Med. Sciences 1997; 313 (5): 283–288.

[16]Dambrin C, Klupp J, Morris RE. Pharmacodynamics of immunosuppressive drugs. Current Opinion in Immunology 2000; 12: 557–562.

[17] Kobashigawa JA. Mycophenolate mofetil in cardiac transplantation. Current opinion in Cardiology 1998; 13: 117 – 121.

[18] Perico N, Remuzzi G. Prevention of transplant rejection. Current treatment guidelines and future developments. Drugs 1997; 54(4): 533 – 570.

[19] Fancois M, Büchler M, Halimi JM, Al-Najjar A, Valentin JF, Thibault G, Lebranchu Y. Lymphocytes subsets in renal transplant recipients treated with mycophenolate mofetil. Transplant Proc 2000; 32: 2781–2782.

[20] Hu H, Dong Y, Feng P, Fechner J, Hamawy M, Knechtle SJ. Effect of immunosuppressants on T-cell subsets observed in vivo using carboxy-fluorescein diacetate succinimidyl ester labeling. Transplantation 2003; 75(7): 1075-7.

[21] Muller TF, Grebe SO, Neumann MC, Heymans J, Radsak K, Sprenger H, Lange H. Persistant long-term changes in lymphocyte subsets induced by polyclonal antibodies. Transplantation 1997; 64: 1432–1437.

[22] Brown MW, Bolton EM, More IAR, Bradley JA. Immunohistochemical observations of rat fetal pancreas allografts transplanted into unmodified and cyclosporine-treated recipients. Transplantation 1988; 46(6): 800–806.

[23] Schulak JA, Masih R, Krishnamurthi V, Robinson A. Mycophenolate mofetil immunosuppression in rat pancreas allotransplantation. J Surg Research 1996; 60: 79–83.

[24] Bihl G. Immunosuppresssion in solid organ transplantation and beyond. The Specialist Forum 2004; 4(3): 28 –40.

[25] Pelegrí C, Morante MP, Castellote C, Castell M, Franch A. Administration of a nondepleting anti-CD4 monoclonal antibody (W3/25) prevents adjuvant arthritis, even upon rechallenge: parallel administration of a depleting anti-CD8 monoclonal antibody (OX8) does not modify the effect of W3/25. Cell. Immunol. 1995; 165: 177-182.

[26] Sablinski T, Hancock WW, Tilney NL, Kupiec-Weglinski JW. CD4 monoclonal antibodies in organ transplantation--a review of progress. Transplantation 1991; 52(4): 579-89.

[27] Kenny E, Mason D, Pombo A, Ramirez F. Phenotypic analysis of peripheral $CD4^+CD8^+$ T-cells in the rat. Immunology 2000; 101: 178–184.

[28] Ilano AL, Mcconnell MV, Gurley KE, Spinelli A, Pearce NW, Hall BM. Cellular basis of allograft rejection in vivo. V. Examination of the mechanisms responsible for the differing efficacy of monoclonal antibody to $CD4^+$ T-cell subsets in low- and high-responder rat strains. J Immunol 1989; 143(9): 2828-36.

[29] Stumbles P, Mason D. Activation of CD 4^+ T-cells in the presence of a non-depleting monoclonal antibody to CD 4 induces a Th 2- type response in vitro. J Exp Med 1995; 182: 5-13.

[30] Qi Z, Riesbeck K, Östraat Ö, Tufveson G, Ekberg H. Single dose anti-CD4 monoclonal antibody for induction of tolerance to cardiac allograft in high- and low-responder rat strain combinations. Transpl. Immunol. 1997: 5: 204-211.

[31] Billingham RE, Brent L, Medawar PB. Actively aquired tolerance of foreign cells. Nature 1953; 172: 603.

[32] Pearson TC, Madsen JC, Larsen CP, Morris PJ, Wood KJ. Introduction of transplantation tolerance in adutlts using donor antigen and anti-CD4 monoclonal antibody. Transplantation 1992; 54(3): 475-483.

[33] Bushell A, Morris PJ, Wood KJ. Induction of operational tolerance by random blood transfusion combined with anti-CD4 antibody therapy. A protocol with significant clinical potential. Transplantation 1994; 58(2): 133-9.

[34] Saitovitch D, Morris PJ, Wood KJ. Recipient cells expressing single donor MHC locus products can substitute for donor-specific transfusion in the induction of transplantation tolerance when pretreatment is combined with anti-CD4 monoclonal antibody. Evidence for a vital role of $CD4^+$ T cells in the induction of tolerance to class I molecules. Transplantation 1996; 61(10): 1532-8.

[35] Stumbles P, Mason D. Activation of $CD4^+$ T-cells in the presence of a non-depleting monoclonal antibody to CD4 induces a Th2-type response in vitro. J. Exp. Med. 1995; 182: 5–13.

[36] Cobbold SP, Qin S, Leong LY, Martin G, Waldman H. Reprogramming the immunesystem for peripheral tolerance with CD4 and CD8 monoclonal antibodies. Immunol. Rev. 1992; 129: 165–201.

[37] Kataoka M, Shimizu Y, Margenthaler JA, Landeros K, Otomo N, Flye MW. Transfer of "infectious" cardiac allograft tolerance induced by donor-specific transfusion. Surgery 2002; 132(2): 167–172.

[38] Hamano K, Ohmi K, Esato K, Fukumoto T. Effect of donor specific transfusion (DST) is restricted to the rat combinations used and closely related with alloantibody in heart transplantation. Immunol Cell Biol. 1989; 67: 297–302.

[39] Tchervenkov JI, Cofer BR, Davies C, Alexander JW. Indefinite allograft survival induced by the combination of multiple donor-specific transfusions, cyclosporine, and an anti-T cell monoclonal antibody in a protocol relevant to cadaveric organ transplantation. Transplantation 1995; 59(6): 821–824.

[40] Frede SE, Valente J, Alexander JW, Babcock GF. The relationship of blood transfusion and immunosuppression to the Th1/Th2 paradigm. Transplant Proc 1997; 29: 1153-1154.

[41] Roelen DL, Bushell AR, Niimi M, Young NT, Rust NA, Morris PJ, Wood KJ. Immunoregulation by CD4 T-cells in the induction of specific immunological unresponsiveness

to alloantigens in vivo: evidence for a reduction in the frequency of alloantigen-specific cytotoxic T-cells in vitro. Human Immunol 1998; 59: 529– 539.

[42] Niimi M, Shirasugi N, Ikeda Y, Kan S, Takami H, Hamano. Importance of thymus to maintain operational tolerance to fully allogeneic cardiac grafts. Ann Thora. Surg 2001; 72: 735–739.

[43] Rakieten N, Rakieten ML,Nadkarni MV. Studies on the diabetogenic action of streptozotocin. Cancer Chemoth.Rep. 1963; 29: 91-98.

[44] Junod A, Lambert AE, Stauffacher W, Renold AE. Diabetogenic action of streptozotocin: relationship of dose to metabolic response. J. Clinical Invest. 1969; 48(11): 2129-39.

[45] Juang J-H, Hsu BR-S, Kuo C-H, Huang H-S. Normoglycemic environment is important for the growth and function of islet isografts. Transplant Proc 1998; 30: 565 – 566.

[46] Caluwaerts S, Holemans K, van Bree R, Verhaeghe J, Van Assche FA. Is low-dose streptozotocin in rats an adequate model for gestational diabetes mellitus? J. Soc. Gynecol. Investig. 2003; 10(4): 216-21.

[47] Brown J, Heininger D, Kuret J, Mullen Y. Islet cells grow after transplantation of fetal pancreas and control of diabetes. Diabetes 1981; 30: 9-13.

[48] Mullen YS, Clark WR, Molnar IG, Brown J. Complete reversal of experimental diabetes mellitus in rats by a single fetal pancreas. Science 1977; 195: 68.

[49] Spence RK, Perloff LJ, Barker CF. Resident's corner. Fetal pancreas in treatment of experimental diabetes in rats. Current Surgery 1981; 38: 138-140.

[50] Kemp JA, Mullen Y, Weissman H, Heininger D, Brown J, Clark WR. Reversal of diabetes in rats using fetal pancreas stored at –196 °C. Transplantation 1978; 26(4): 260–264.

[51] Eloy R, Doillon C, Dubois P. Fetal pancreatic transplantation – Review of experimental data. Transplant Proc 1980; 12(4): 179–185.

[52] Suylichem PTR, Strubbe JH, Houwing H, Wolters GHJ, Van Schilfgaarde R. Insulin secretion by rat islet isografts of a defined endocrine volume after transplantation to three different sites. Diabetologia 1992; 35: 917-923.

[53] Hiller WFA, Klempnauer J, Lück R, Steiniger B. Progressive Deterioration of Endocrine Function After Intraportal but Not Kidney Subcapsular Rat Islet Transplantation. Diabetes 1991; 40: 134-140.

[54] Brown J, Clark WR, Molnar IG, Mullen YS. Fetal pancreas transplantation for reversal of streptozotocin-induced diabetes in rats. Diabets 1975; 25(1): 56-64.

[55] Spence RK, Perloff LJ, Barker. Fetal pancreas in treatment of experimental diabetes in rats. Curr Surg 1981; 38: 138-140.

[56] Adeghate E. Pancreatic tissue grafts are reinnervated by neuro-peptidergic and cholinergic nerves within five days of transplantation. Transpl Immunol 2002; 10: 73 –80.

[57] Muller CJF, Du Toit DF, Page BJ, Louw J, Muller BJF, Williams K, Mattysen JJ, Lyners RFC. Islet morphology and function following foetal rat pancreatic transplantation. Eur J Anat 2000; 4(3): 149-160

[58] McEvoy RC, Hegre OD. Syngeneic transplantation of fetal rat pancreas. Diabetes 1978; 27(10): 988-995.

Chapter 9

CONCLUSIONS

AND

FUTURE AREAS OF RESEARCH

CONCLUSIONS.

1. Isogeneic foetal rat pancreatic transplantation under the kidney capsule allows for the development and differentiation of endocrine tissue (islets of Langerhans) which is capable of reversing and stabilizing the clinical and gross metabolic derangements of experimentally induced diabetes in a laboratory rat model. Normoglycaemia is thereby ensured following isogeneic FRPT in this model, provided sufficient foetal tissue is engrafted.

2. Following engraftment of isogeneic foetal rat pancreatic tissue under the kidney capsule, the endocrine tissue develops while the exocrine tissue component atrophies. Morphologically, the engrafted islets of Langerhans appear normal, although immunocytochemistry shows a decrease in the non-β cell islet component which could be due to a lack of growth factors in the renal subcapsular environment or some form of negative feedback from the existing host pancreatic cells. Functionally, these grafts do secrete insulin and are able to maintain normoglycaemia in STZ induced diabetic rats.

3. Allogeneic foetal rat pancreatic grafts, across weak or strong histocompatibility barriers, are rapidly rejected and destroyed unless immunosuppressive therapy is instituted that not only prevents rejection but also allows for growth and development of the foetal grafts into mature endocrine tissue. Non-immunesuppressed pancreatic allografts are consistently rejected within a period of 14 days.

4. Flow cytometry shows that immunosuppressive agents that effectively target $CD4^+$ T-cells and result in a significant decline in $CD4^+$ peripheral blood percentage can predictably prevent or delay allograft rejection. By combining specific immunosuppressive agents that target different T-cell activation cascade molecules i.e. anti-CD4 monoclonal antibodies, cyclosporine and mycophenolate mofetil, the suppressive effect on the $CD4^+$ T-cells is enhanced

5. $CD4^+$ T-cells are rapidly removed or migrate from the peripheral blood circulation following the administration of a mouse anti-rat CD4 monoclonal antibody (W3/25). Although W3/25 is a non-depleting antibody, we have demonstrated that, by removing circulating $CD4^+$ lymphocytes from the peripheral blood circulation, it acts in a similar manner to a depleting antibody. Withdrawal of the antibody leads to the reappearance of the circulating $CD4^+$ lymphocytes.

6. Rejection of allogeneic foetal rat pancreatic transplantation can be consistently prevented, or delayed, by targeting the CD4 molecule with a mouse anti-rat CD4 monoclonal antibody (W3/25). A light to moderate graft infiltrate, which includes $CD4^+$ and $CD8^+$ lymphocytes, suggests a measure of peripheral immunological unresponsiveness/suppression which results in graft acceptance.

7. Cyclosporine (CsA), given daily as monotherapy or in combination with mycophenolate mofetil prevents rejection of allogeneic FRPT and allows for the development and maturation of endocrine tissue (islets of Langerhans).

8. Mycophenolate mofetil, as a monotherapy, failed to prevent rejection of allogeneic FRPT in these allogeneic models.

9. W3/25 administered as daily doses of 500 μg per day, prevented graft rejection for 14 days in the SD rat. However, by 30 days post-transplantation, one rejection (1/6) had occurred. In the PVG rat, W3/25 monotherapy was less effective with rejections occurring 14 and 30 days post-transplantation. These findings illustrate the importance of rat models used in transplantation, especially when using antibody therapy. The differences in the results between these two rat strains could be due to various strain specific factors such as the ability of $CD8^+$ lymphocytes to initiate rejection without the help of $CD4^+$ lymphocytes or direct alloantigen recognition.

10. By combining W3/25 and cyclosporine a synergistic effect results, which effectively prevents rejection of FRPT across strong responding rat strain histocompatibility barriers.

11. A single donor specific transfusion (DST), combined with 5 consecutive daily doses of W3/25 plus CsA induction prior to transplantation, resulted in graft unresponsiveness for a 30 day period without the need for any further daily immunosuppression. This induction protocol not only effectively prevents rejection, but also results in excellent growth and development of endocrine tissue from allogeneic foetal rat pancreata. This finding is of great importance to transplantation immunologists as similar immunosuppressive protocols are applicable in the clinical situation. This was one of the cardinal aims of this dissertation.

12. The clinical signs of streptozotocin-induced diabetes were successfully reversed by allogeneic FRPT immunesuppressed with DST, CsA and W3/25 induction therapy. To my knowledge, this is the first study in which streptozotocin induced diabetes could be effectively reversed in a fully MHC mismatched allogeneic rat model.

13. The end-points and desired outcomes of the research have been consistently achieved. Hyperglycaemia has been consistly reversed and normoglycaemia restored in an isogeneic and an allogeneic diabetes rat model by foetal rat pancreatic transplantation. Utilization of combination therapy including DST, W3/25 and CsA proved to be highly effective in suppressing rejection-effector mechanisms.

FUTURE AREAS OF RESEARCH.

This foetal rat pancreatic transplantation model offers several exciting and challenging areas for further research. The small alloantigen load associated with FRPT and the growth potential makes this model ideal for evaluating the toxic side effects of the new generation of immunosuppressants including tacrolimus, sirolimus, everlimus and MNA.

The metabolic effect of immunosuppressants, and other pharmaceutical agents, on developing foetal pancreas transplant, can be established and monitored by insulin and blood glucose determinations in this experimental diabetic model.

Intragraft immunology is another field of transplantation biology worth pursuing. The cell immunophenotypes, function and cytokine profiles, both during rejection and in the unresponsive allograft, need to be elucidated as these data could hold the key for unlocking the mechanisms of the ultimate goal in transplantation, namely the establishment of predictable tolerance.

The model offers the embryologist an opportunity to study the endocrine development of the islets of Langerhans in an ectopic environment, which will contribute greatly to our knowledge of islet transplantation. Issues such as revascularization, reinnervation and the exocrine endocrine relationship are also important areas for future research.

APPENDIX A

RESEARCH PROTOCOL: PROPOSAL FOR A Ph.D. DEGREE: REGISTRATION DURING 2000.

RESEARCHER: MR.C.J.F.Muller (Curriculum vitae attached).

DEPARTMENT: Anatomy and Histology, Tygerberg Campus.

PLACE OF RESEARCH: Department of Anatomy and Histology Research Laboratory and the Department of Medical Biochemistry, Faculty of Medicine, University of Stellenbosch.

PROPOSED PROMOTOR AND

SUPERVISOR OF RESEARCH: Professor Don du Toit. Department of Anatomy and Histology, Faculty of Health Sciences, Tygerberg Campus, University of Stellenbosch.

CO-PROMOTOR: Professor P.J.D. Bouic. Department of Microbiology and Immunology, Faculty of Health Sciences, Tygerberg Campus, University of Stellenbosch.

CLASSIFICATION OF RESEARCH: Experimental animal bioresearch

THEME OF RESEARCH: Diabetes mellitus / transplantation / histology / immunology / endocrinology / rejection.

TITLE OF RESEARCH PROJECT: Preclincal assessment of the novel immunosuppressive properties of anti-CD4 Monoclonal antibodies (mAb) in an allogeneic foetal rat pancreatic transplantation, diabetic, laboratory animal model.

CURRENT PROTOCOL REGISTRATION WITH SUB-COMMITTTEE-C/ETHICAL COMMITTEE.

1.1 Title: "In-vivo and in-vitro studies with human foetal pancreas: preclinical studies". The study has been in progress since early 1995 and reregistered during 1995 – 2000.

1.2 Registration No: 87/072

1.3 The researcher wishes to concentrate on the animal experimentation portion of the protocol and possibly use the human slant at a later stage for which permission will be sought.

STATISTICAL PLANNING: Some of the tissue processing work has been performed in collaboration with the Medical Research Council for the past 4 months where computerized parametric and non-parametric analyses are currently being applied. Careful planning has been made to address the specific interpretation of the histological, glucose and insulin tolerance parameters. PC-Shareware statistical package/Statman(for means, medians, variance, standard deviation, range, descriptive statistics). Statistica for the determination of significant differences. In addition EC-Stat, multivariate statistical assessment is to be applied together with the multivariate statistical package (MVSP), Epistat and Statgraphics, which is available on the network. Specific attention has been payed to correct sample size, controls and avoidance of type-II errors.

RESEARCH FUNDING: Grant awarded from the Harry Crossley Fund: November 1999 for protocol number 87/072.

INFRASTRUCTURE: The histology laboratory and theatre facilities in the Department allow the following techniques and procedures:

1.1 All microsurgical procedures/transplants/dissection.

1.2 Histological tissue processing at light and EM levels.

1.3 Staining capacity for routine H&E, EM, specialized light microscopy stains and immunocytohistochemistry (i.e. Immunoperoxidase).

1.4 Metabolic facilities to assess parameters of diabetes.

1.5 The infrastructure is of a high standard and sufficient to enable the completion of the experimental work. Excellent infrastructure is present in the Central Animal Research Unit and the Department of Medical Physiology and Biochemistry to raise the monoclonal antibodies in mice and perform the refining process.

RESEARCH STATEMENT: The current research is an advanced, original theme/approach based on laboratory-directed, animal research/methodology and is aimed at contributing to the fundamental and theoretical knowledge (endocrine, histological and immunological) and understanding of diabetes mellitus specifically by attempting to surgically ameliorate the

hyperglycemia of chemically-induced diabetes mellitus (by Streptozotocin), by the sophisticated microtransplantation of foetal pancreatic tissue in diabetic inbred and allogeneic rats. The study of the use of novel monoclonal antibodies (mAb) such as clone W3/25 (Biological agent) alone or in combination with corticosteroid induction therapy, to suppress the rejection effector mechanisms responsible for graft destruction (as foetal pancreatica) has not been reported. The use of this new biological immunesuppressant will be a very valuable contribution to the current literature on anti-rejection therapy and immune-biology. This form of unique, sophisticated research with very strong clinical application/slant is only performed in a very few select laboratories in the world and in no unit in RSA. The potential of eventually being able to combine a biological immunosuppressant such as W3/25 or other with chemical immunosuppression such as corticosteroids or cyclosporine (CsA) may have far reaching positive benefits for transplant recipients with the added possibility of inducing specific immune tolerance. Thus, the problem in medicine being addressed in this study is the amelioration of chemically-induced diabetes mellitus in a diabetic rat model with the objectives of the transplantation of viable, foetal pancreatic tissue with potential to grow and differentiate across a strong histocompatibility barrier (i.e. allogeneic barrier), thus allowing partial or complete restoration of normoglycaemia apart from the other possible advantages, forthcoming to the disturbed metabolism.

STATEMENT OF ORIGINALITY: The following aspects of the research endeavours make the project original and unique.

1.1 Definition of the role of the CD4 lymphocyte in the rejection of MHC mismatched foetal rat pancreatic allografts in diabetic rats, assessment of the pharmacokinetics and modulation of the CD4 surface receptor molecule following the use of mAbs and characterization of the mononuclear cellular infiltrate in the foetal grafts by the use of immunofluorescent labeling (CD2, CD4 and CD8).

1.2 Combination of anti-CD4 mAb with high dose corticosteriod induction therapy to enhance a Th2 response to allogeneic antigen is an unique research slant not described in the literature.

1.3 Comparison and correlation of histological morphometric parameters of foetal pancreatic tissue organogenesis at immunocytohistochemical (i.e. maturation and development of the islets of Langerhans) level with specific reference to A, B, D and PP-cells in allogeneic diabetic recipients.

ETHICAL ASPECTS: Rigid ethical code conduct has been adhered to according to the "Ethical Considerations in Medical Research Revised Edition MRC" – pages 34 – 39 pertaining to animal research since registration of protocol 87/072. In particular the proper use of painkillers, care and the administration of anaesthesia according to recognized veterinary practice has enjoyed high priority. As far as anaesthesia is concerned in rats, this laboratory has found the use of ether, thiopentone, Ketalar®, local anaesthesia and chloral hydrate (either singly or in combination) completely adequate for the type of specific procedure needed in this project. Postoperative warmth is provided by short-term overhead lighting, which also reduces immediate

postoperative pain. A register of experimentation reflecting animal number, procedure and outcome is kept and available for inspection.

NEED FOR THIS SPECIFIC STUDY: The motivation for undertaking this study and the following circumstances prompted the research in this field.

1.1 Diabetes, apart from cardiovascular disorders, trauma and cancer is one of the leading causes of premature death in man, Thousands of papers on the disorder continue to appear yearly in medical journals as cited in the Index Medix and Scientific Index. The papers report on the controversial aspects of aetiopathogenesis, metabolic and endocrine abnormalities and treatment of diabetes.

1.2 The possibilities of treating diabetics, once rejection is controlled, by foetal implantation of endocrine tissue such as foetal islets is very exciting.

1.3 The prevalence of diabetes mellitus is increasing rapidly worldwide and in South Africa afflicts many underprivileged population groups.

1.4 Biological immunesuppressants such as mAbs, very importantly, do not show the diabetogenic potential induced by cyclosporine (CsA), steriods and FK506 (Tacrolimus) and are specific in their mechanism of action thus making it important to pursue this line of research.

1.5 The adverse side effects seen after conventional immunosuppression, necessitates a modified approach to immunosuppression. This study, by using monoclonal antibodies

against epitopes of cell surface receptor molecules, associated with alloantigen recognition, will hopefully improve our understanding of the graft rejection process and immunosuppression. This would not only benefit diabetic patients receiving transplants but organ transplantation in general.

PROBLEM ADDRESSED: The following key research aspects are being addressed:

1.1 Inhibition or suppression of specific rejection effector mechanisms (T-cell generated) by the use of anti-CD4 mAb (in this case W3/25, a mAb obtained from the University of Oxford, UK), alone or in combination with high dose steriod induction therapy, in a allogeneic foetal rat pancreatic transplantation laboratory model.

1.2 Restoration of normoglycaemia in diabetic rats (induced chemically by streptozotocin. A known long-term B-cell specific toxin in adult rats) by allogeneic transplantation of foetal rat pancreata across a MHC barrier and inhibition of rejection by the use of mAb.

1.3 Correction and study of the clinical and some of the metabolic abnormalities (i.e. ketosis) induced by chemical diabetes in rats by allogeneic foetal rat pancreatic endocrine islet-cell replacement.

END-POINTS OF RESEARCH IN THIS MODEL: The following end-points of the research have been defined:

1.1 Reversal of hyperglycaemia by syngeneic and allogeneic FRPT in diabetic rats. Induction of random WBG <10 mmol/l.

1.2 Reversal of the clinical signs of diabetic rats by syngeneic and allogeneic FRPT (ketosis, polyria, polydipsia, glycosuria).

1.3 Medium to long-term (30 – 90 days) suppression of rejection by mAbs (as evaluated by graft histology at light and EM level) with the preservation and maturation of islets of Langerhans in allogeneic recipients.

PREAMBLE: The ultimate purpose, goals and clinical application of pancreatic transplantation in diabetic patients are two-fold:

1.1 To establish a continuous normoglycaemic, insulin-independent state in a diabetic recipient and to induce a permanent state of euglycaemia in insulin dependant diabetics.

1.2 To prevent, halt or reverse progression of the secondary complications of diabetes in the long-term, thus improving the quality of life in the diabetic recipient. Early studies in man have shown that some of these objectives are achievable if the complications are not irreversible.

BRIEF LITERATURE REVIEW: Diabetes – the problem, place of foetal pacreatic transplantation, and use of monoclonal antibodies as immunesuppressants.

Briefly, the clinician knows and treats two types of "Diabetes" i.e. Type I (IDDM) and Type II (NIDDM). Both conditions run in families, are associated with accelerated atherosclerosis,

microangiopathy resulting in retinopathy, nephropathy and neuropathy. The risk of stroke is doubled: the risk for heart attacks are increased 2-3-fold, there is a 50-fold prevalence in the incidence of peripheral vascular disease. Finally, life expectancy is significantly shortened in both males and females (Joslin), and in the USA the disease kills several hundred thousands due to its effects on small blood vessels. Treatment is directed at correcting dietary deficiencies, weight reduction, prevention of infections, application of newer methods of insulin delivery and accurate blood glucose monitoring by portable glucometers. Despite this, secondary complications still occur and are particularly common in underprivileged third-world nations and races. Diabetics remain uninsurable by insurance companies or pay enormous premiums. The cost of insulin is staggering and most medical-aids refuse cover for diabetics, even if excellently controlled.

Apart from the conventional medical research in the field of diabetes, the Pancreas Transplant Registry of June 1995 shows that 5546 pancreas transplants have been engrafted into diabetics worldwide over the last 7- years. Precise indications for transplantation now exist, but are mainly preformed on IDDM subjects with complications. USA statistics (of June 1995) show posttransplantation survival of 80% at 60 months and graft survival of 60% at 60 months. One-year graft survival of whole grafts is about 74%. In all cases the patient is committed to life-long cyclosporine based immunosuppressive treatment. Recurrence of the diabetes due to autoimmune destruction of the graft has been shown in extensive studies not to be a big clinical problem as the patients are committed to long-term immunesuppression. Islet and foetal transplantation have also become popular modes of transplantation and are gaining momentum. Human clinical islet transplantation results has despite early optimism been disappointing. 305 adult islets allografts were preformed at 38 institutions by December 1995 of these patients only

39 were insulin dependant at 1 month of which only 24 were still insulin dependant at 12 months and only 1 patient is still insulin dependant at 4 years (International transplant registry, 1996, 6). Analysis c-peptide negative patients receiving islet transplants, in the period 1990 – 94, showed that of 96 patients know to be c-peptide negative prior to transplantation, 27% had basal c-peptide levels in excess of 1ng/ml at 1 year and of these only 7% were insulin dependant (International transplant registry, 1996, 6). These patients with persisting c-peptide levels, although not sufficient to be insulin independent, benefit with lower doses of insulin and improved glycemic control and thus less complications (Weir Diabetes vol 46 1247 –56). More specific to this study, foetal tissue has been transplanted to the subrenal capsule site (favored site) in man and rodent models by a few other researchers abroad.

Successful engraftment of foetal tissue into the liver, spleen and muscle has been less successful for a variety of reasons. The main motivation for foetal transplantation is that foetal tissue has the capacity to grow, differentiate and function and to control diabetes when transplanted into athymic mice. Lafferty has shown growth and function in man following subrenal capsule transplantation in diabetic patients by demonstrating a sustained reduction in insulin requirement and elevation of basal C-peptide levels in IDDM recipients. Once the foetal pancreas is removed from the donor and transplanted, the exocrine component degenerates allowing the endocrine aspect to differentiate into islets with their own blood supply and capsule. In rodents only two foetal pancreases per adult rat are needed to consistently reverse streptozotocin-induced diabetes. Reversal takes about 2-3 weeks. The exuberant proliferation potential and ease and safety of engraftment in safe, accessible sites in diabetic rodents, with the ultimate potential of human application, promoted the study, the only of its kind in South Africa.

Although the discovery of insulin by Banting and Best has lead to a great improvement of both life expectancy and quality of life in the diabetic, it soon became clear that the diabetic is faced with many disease related complications. Although there is a good correlation between tight diabetic treatment control as measured by a glycaemic index, complications like retinopathy, nephropathy and neuropathy still progress although at a slower rate. Sudden changes in diabetic control even for the better may accelerate the progression of the disease. Prediction of patients predisposed to developing these often life-treating complications is normally not possible although patients expressing the HLA-DR3 and –DR4 genotypes show an increased risk (Tarn).

Successful pancreas transplantation, as a treatment option, has been shown to slow or even reverse the onset of diabetes related complications. This is especially true for nephropathy reoccurring in a transplanted kidney. Reversal of chemically induced diabetes in mice and rats by foetal pancreas transplantation is possible and euglycemia can be maintained for long periods provided that adequate immunosuppression is maintained (Brown). Conventional immunosuppressants, like cyclosporine (CsA) are effective in preventing both graft rejection and suppressing the autoimmune process provided that the treatment is maintained. The nephrotoxic effect of drugs like CsA combined with the high incidence of nephropathy in diabetic patients contraindicates against prolonged use of these drugs have a direct adverse effect on islet function (Weir). Depletion of CD4+ lymphocytes using a complement fixing monoclonal antibody directed at the CD4 cell surface receptors could induce indefinite survival of MHC incompatible pancreas allografts in mice (Ilano). Depletion of CD4 lymphocytes renders the animal highly immune compromised. Recent reports have shown that complete depletion of the CD4 lymphocytes is not necessary and that non-depleting anti CD4 monoclonal antibodies like YTS 177.9 in mice and RIB 5/2 in rats can induce allograft unresponsiveness while preserving the

immune system (Lehmann). The use of monoclonals to prevent graft rejection could be very appropriate in pancreas allografts since it does not produce the unwanted side effects associated with chemical immunosuppressive drugs. W3/25 a non-depleting rat anti-CD4 mAb recognizing a different epitope to RIB 5/2 has been used to prevent the induction and progression of various autoimmune diseases but its efficacy in preventing allograft rejection has not been established (Mannie). W3/25, a rat anti-CD4 clone, has a strong inhibitory effect on mixed lymphocyte reactions and has been shown to shift the immune response from a T-helper 1 to a T-helper 2 like response by suppressing IFNγ (a cytokine associated with rejection) production and enhancing IL-4 and IL-13 (cytokines associated with graft survival) secretion, provided W3/25 was administered during the primary exposure of the allograft antigen (Webb and Masson). A short high dose course of corticosteroids at the time of transplant (introduction of antigen) has a similar TH-2 promoting effect. The combination of anti-CD4 mAb with corticosteroid pulse therapy, to preferentially induce an tolerogenic TH-2 response, has not been described in the literature and provides an original area of research. Clonal development tolerogenic, TH-2 promoting CD4 cells can be distinguished flow cytometrically from TH-1 promoting CD4 cells by their expression of CD45RC (OX22). Following antigen exposure these tolerogenic CD4 cells express low levels of CD45RC molecules while Th1 effector cells have high levels of CD45RC. It is therefore possible to monitor the establishment and clonal expansion of tolerogenic/suppressor cells following different treatment protocols. This approach has direct clinical implications as the same regulatory CD4 subtype has been described in man (Mason. Immunlogy 1990).

HYPOTHESES:

1. Transplanted foetal islet-containing pancreatic tissue undergoes selective histological differentiation and islet maturation in-vivo after engraftment thus allowing amelioration of hyperglycaemia of chemically induced diabetes (Streptozotocin) in allogeneic recipients across a strong histocompatibility barrier.

2. Foetal rat pancreatic transplantation (FRPT) is capable of partially or completely reversing the clinical and metabolic aberrations in diabetic rats.

3. Anti-CD4 monoclonal antibodies (such as W3/25) administered to allogeneic recipients of foetal rat pancreatic grafts in a strong responding model are capable of preventing or suppressing acute rejection of the transplanted tissue.

4. The action of anti-CD4 can be further enhanced by high dose corticosteroid induction therapy.

FEASIBILITY AND PILOT STUDY 1996/7: A feasibility and pilot study has been completed with success during 1996/7 and confirms that the proposed research project is viable, the diabetic rat model is established, surgical techniques are possible and that the laboratory infrastructure for the sophisticated staining of tissues, production and refining of W3/25 is in existance and functioning. The study shows that successful engraftment of foetal pancreas beneath the renal capsule is possible allowing proliferation and growth of the transplanted foetal tissue. Reversal of chemical induced diabetes by streptozotocin is also capable in syngeneic recipients (WAG ⇒ WAG; substrain of Wistar and is inbred or isogeneic). The pilot study was further expanded to establish the viability of the immunological arm of the project. WAG (a substrain of Wistar rats) foetal pancreases of between 18 and 20 days gestation were transplanted under the kidney

capsule of adult Spraque- Dawley rats. The recipients were then randomized into the following groups:

Group 1. Control group (n = 4) received no immunosuppression (unmodified rejection).

Group 2. CsA group (n = 5) received cyclosporine (CsA) 2 mg/kg/d (imi)

Group 3. W3/25 group (n = 5) received W3/25 200 μg/d (ipi)

Group 4. W3/25 group (n = 5) received W3/25 500 μg/d (ipi)

All animals in groups 1,2 and 3 were sacrificed at 10 days. The animals in group 4 were treated for 50days and then sacrificed. Results showed fierce uncontrolled rejection of foetal pancreas allografts in the control group. This confirms the strength of the histocompatibility mismatch and suitability of the model. Immunosuppression for 10 days using W3/25 or CsA 2mg/kg prove effective in preventing graft rejection and allowing development of islets from the foetal pancreas allografts. Two of the animals in group 4 showed substantial graft preservation while the other had rejected their grafts. These encouraging results show that W3/25 is capable of preventing graft rejection in the short and longer term. Results were presented as an publication in an international peer-reviewed journal and in the form of oral presentations which were well received at the following international and national congresses:

Publication

1. Prolongation of Rat Fetal Pancreas Allograft Survival Using a Nondepleting Anti-CD4 Monoclonal Antibody W3/25. CJF Muller, DF du Toit, AD Beyers BJ Page and N Muller. Transplant Proceedings 1998, 30 (8): 4180–4183.

Congress presentations

1. Does the Endocrine component of the Foetal Rat Islet Change Following Syngeneic and Allogeneic Transplantation and Suppression with Combinations of Anti-CD4 mAb, CsA and DST? Du Toit DF, Muller CJF, Mouton YM, Lyners RFC, Muller N, Wessels J, Fourie P, Beyers AD and Woodroof C (In association with the Medical Research Council). XVII Congress Of The Southern African Transplantation Society, Stellenbosch March 1997.

2. Characterization Of The Intragraft Mononuclear Cell Infiltrate After Sub-Capsular Allogeneic Foetal Pancreas Transplants In Rats. Muller CJF, Du Toit DF, Mouton YM, Muller N and Beyers AD. 27th Congress of the Anatomical Society of Southern Africa Cape Town 15 - 18 April 1997.

3. A Comparison of Foetal Pancreas Allograft Immunemodulation in Rats, using a Non-depleting Anti-CD4 Monoclonal Antibody (W3/25) to Cyclosporin. Muller CJF, Du Toit DF, Mouton YM and Beyers AD. 37th annual congress of the federation of Southern African Society of Pathology, Cape Town 2 July 1997.

4. Prolongation of Rat Foetal Allograft Survival using a Novel non-depleting Rat Anti-CD4 Monoclonal antibody W3/25. Muller CJF, Du Toit DF, Beyers AD, Mouton YM, Muller

N, Lyners RFC and Mattysen J. Surgical Research Society of Southern Africa Congress, 17 –19 July 1997, Stellenbosch.

5. Prolongation of Foetal Rat Pancreas Allograft Survival using a Novel non-depleting Rat Anti-CD4 Monoclonal Antibody Clone W3/25. Muller CJF, Du Toit DF, Page BJ, Beyers AD, Muller N, Mattysen J and Lyners RFC. 3rd International Conference On new Trends In Clinical And Experimental Immunosuppression, 12 – 15 Februarie 1998, Geneva, Switzerland.

6. Prolongation Of Foetal Rat Pancreas Allograft Survival Using A Nondepleting Anti CD4 Monoclonal Antibody W3/25. CJF Muller, DF duToit, AD Beyers, BJ Page, N Muller, J Mattysen, and R Lyners. XVIII Congress Of The Southern African Transplantation Society. 18 - 21 April 1999. Mount Amanzi Lodge, Hartebeesport Dam.

PROPOSED RESEARCH PROGRAM - Research Aims and Objectives:

A: Immunological and Endocrine Studies:

1.1 To define the role of the CD4+ lymphocyte in the rejection of MHC mismatched allografts with particular reference to foetal pancreas transplantation in the rat.

1.2 To modulate the CD4 surface receptor molecule using monoclonal antibodies directed against different epitopes of the CD4 molecule, and to study the pharmacokinetics following the administration of the antibodies.

1.3 To combine anti-CD4 mAb therapy with short high dose corticosteriod induction therapy at the time of antigen exposure.

1.4 To study the immunological response following engraftment and mAb treatment using both in vivo (skin grafts) and in vitro (lymphocyte activation markers CD25).

1.5 To study the antagonistic or synergistic effect of combining CD4 mAb treatment with other co-signal producing membrane surface receptors like TCR, CD2, CD5 and CD28 in the modulation of graft rejection.

1.6 Assessment of the efficacy of anti-CD4 mAbs to suppress or prevent rejection of foetal allografts in the MHC mismatched DA (Dark Arguti) ⇒ PVG transplant model.

1.7 Assessment of the capability of allogeneic foetal rat pancreatic allografts to reverse the hyperglycaemia and other clinical features of chemically induced diabetes.

1.8 Evaluation of the endocrine response post-transplantation (IVGGT).

B: Histological Studies:

Assessment of the morphology of the developing foetal rat pancreas (18 - 21) day, post-coitum, time-bred pregnant foetal pancreases.

Histological assessment of the islet-cell morphology after allogeneic transplantation.

2.1 Light microscopy (H&E).

2.2 Special stains i.e. Gomori-Aldehyde fuchsin.

2.3 Immunocytohistochemistry (insulin, glucagon, PP, somatostatin).

2.4 Transmission electron microscopy (ultrastructure).

2.5 Immunofluorescence.

2.6 Flow cytometry.

2.7 Confocal microscope (MRC).

3. Assessment of islet maturation and organogenesis (growth and development) following allogeneic foetal transplantation and immunosuppression with anti-CD4 mAb.

4. Correlation of posttransplantation graft morpholgy and endocrine response or dysfunction.

MATERIALS AND METHODS OF THE PROPOSED STUDY.

Diabetic Model: A standardized laboratory model is utilized. Diabetes is induced by a single tail-vein intravenous injection of Streptozotocin (STZ) at a dose of 45 - 75 mg/kg. Diabetes is present usually within 48 hours and fasting PBG are usually > 15 - 20 mmol/l.

Donor Organs: 19 - 21 day time bred, post-coitum, DA foetal rat pancreata (standardized model). Between 2 - 8 foetal pancreata are transplanted at one sitting into the diabetic recipient after removal from the pregnant female rat. The harvesting of the foetal pancreata occurs via a laparotomy and hysterectomy under general anaesthesia. Thereafter the donor rat is euthanazed

and the procedure becomes a terminal experiment. The tiny grafts are temporarily stored in 4°C RPMI medium (Gibco) before transplantation.

1.3 Transplantation: Under general anaesthesia, between 2 and 8, free, non-cultured, pancreata are transplanted microsurgically under the kidney capsule of the diabetic recipients (renal subcapsular site) and control animals (see groups).

1.4 Method of Anaesthesia in the Rat: General anaesthesia is employed for all procedures performed in the rat and includes anaesthesia for:

(i) Laparotomy and transplantation of pancreatic foetal grafts.

(ii) Harvesting of pancreases.

(iii) Daily venesection for bloodsamples (0.01 ml/d)

(iv) Performance of glucose tolerance tests (IVGTT) (0.01 ml in total for all specimens).

Excellent and adequate levels and depth of anaesthesia are achieved by the use of intraperitoneal administered sodium pentobarbitone or thiopentone (Intraval) - doses (1 - 3 ml ip) for induction together with ether by inhalation for maintenance without the need for a tracheotomy. Special attention must, however, be given to the patency of the airway. The above provides an adequate depth of anaesthesia and suitable anaesthesia, together with a rapid recovery phase. Maintenance of general anaesthesia is enhanced by the addition of Ketalar®(Parke Davis®) given in intramuscular doses of 0.1 - 0.2 ml (of a 5 mg/ml solution) one or two minutes before a procedure is preformed. Ketalar® also has very potent analgesic properties, which is highly suitable for reducing the pain during and after tissue engraftment.

1.5 Immunesuppression: Central to this study is the use of anti-CD4 mAbs (W3/25) which is injected into transplanted allogeneic diabetic control rats via the intraperitoneal route (ipi) on a daily basis (0.1 ml) given under general anaesthesia W3/25 mAb will be prepared personally by the applicant from mouse ascitis, precipitated by $(NH_4)_2SO_4$ and then purified by ion exchange chromatography. Concentration of Ab will be determined by optical density. Appropriate dilutions will be made using sterile PBS. Doses of between 200 - 500 µg/d indefinitely will be studied. Combination of anti-CD4mAb and corticosteriod (methylprednisolone) high dose pulse therapy (10 mg/d IV x 3 days) will further enhance anti-CD4 phamacological action. Control animals receive either no immunesuppression or conventional cyclosporine (CsA) at a determined dose of 2 mg/kg/d indefinitely (0.2 ml) and given intramuscularly (imi).

1.6 Post operative assessment of rejection: The following parameters are used to define rejection:

a. In diabetic transplanted recipients and immunosuppressed with W3/25 or CsA: A re-occurrence of hyperglycaemia after transplantation (i.e. an elevated PLG > 10 mmol/l on two consecutive days).

b. Histological assessment of harvested grafts at various intervals after transplantation at periods of 10, 30, 60, 90 days to show immunological infiltration with lymphocytes and graft destruction. Graft rejection to be scored by the method of Guymer and Mandel (1993).

1.7 Post transplantation Measurements/Parameters Studied:

a. Fasting PLG (measured by an Ames Glucometer (blood obtained from a tail prick - 0.001 ml) Blood glucose determinations are performed daily or every second day until rejection occurs. Rejection is defined as an elevated PLG or WBG . 10mmol/l on two consecutive days.

b. Intravenous glucose tolerance tests (IVGTT) via a standard laboratory technique using 0.5g 50% dextrose and measurement of bloodglucose at 5 minute intervals, after the bolus injection of glucose, for periods of up to 60 - 90 minutes. Insulin will be determined in select animals (Coat-a-count®)

c. Metabolic studies: Performed in specific metabolic cages. Studies include 24-hour determination of weight, water intake, urine output, urine glucose, and urine pH and ketone levels.

d. Pharmacokinetic studies of W3/25 in selected controls using standard methods. Has already been studied in the pilot study.

e. Peripheral blood monitoring (0.01 – 0.02 ml) using a panel of mAb consisting of CD2(OX34), CD4(W3/25), CD8(OX8), CD45RC(OX22), CD38, TCR (R73) and IL2R (OX39) will be analyzed on a FACScan flow cytometer (Becton Dickinson®). In-vivo binding of W3/25 will be monitored using FITC conjugated rabbit anti-mouse IgG.

f. Histological assessment of harvested foetal pancreatic grafts at designated intervals after transplantation to study rejection, graft infiltration, maturation and development of islets of Langerhans especially in immunesuppressed diabetic recipients returned to normoglycaemia. The following methods will be utilized to attain those goals.

- Light microscopy
- Electron microscopy (EM)
- Immunofluorescent labeling
- Immunocytohistochemistry at light and confocal microscopic level – this allows specific identification of A, B, D and PP cells.

g. Posttransplantation goals and end points of research:

- Reversal of hyperglycaemia in diabetic immunesuppressed recipients (measured by daily PBG and urine assessment).
- Lymphocyte reponse to treatment: Measured by flow cytometry of peripheral blood lymphocytes (blood specimen volume needed = 0.02 ml).
- Clinical monitoring of diabetes: Assessment of water intake, urine output, ketones and glucose content.
- Graft rejection: Measured by histology and specific immunocytochemistry and immunophenotyping of CD4 and CD8. Histological scoring by the method of Guymer and Mandel 1993. Characterization of the MNC infiltrate will be

immunologically performed on frozen sections using a panel of antibodies consisting of CD2 (OX34), CD4 (W3/25), CD8 (OX8), CD45RC (OX22), TCR (R73) and IL2R (OX39).

- Graft and animal survival assessed in terms of postgraft days in the three immunesuppressed groups (i.e. W3/25, W3/25 / steriod combination and CsA) by Kaplan-Meier statistics and non-parametric analysis (Spearman-Rank, Mann-Whitney U test).

1.8 Experimental Groups. According to computer statistical randomization. A minimum of 10 animals per group will be used.

- Normal foetal pancreata (histological controls).
- Normal adult rats (DA and PVG): Immunological and endocrine controls.
- Diabetic, non-treated or transplanted controls (for metabolic studies).
- DA ⇒ DA transplantation (inbred, absence of rejection) in diabetic and non-diabetic recipients (to assess organogenesis and growth in the absence of rejection).
- DA ⇒ DA sham controls
- DA ⇒ PVG; no immunesuppression (untreated allograft controls – i.e. unmodified rejection). Diabetic and non-diabetic controls.
- DA ⇒ PVG immunesuppressed with W3/25 (200 –500 μg/d ipi given indefinitely and commencing on day1 of transplantation). Diabetic and non-diabetic controls.

- DA ⇒ PVG immunesuppressed with W3/25 (200 –500 μg/d ipi x28days) combined with methylprednisolone 10mg/d both therapies commencing on day1 of transplantation. Diabetic and non-diabetic controls.

- DA ⇒ PVG immunesuppressed with CsA 2 mg/kg/d imi indefinitely in diabetic and non-diabetic recipients. This group represents the control immunosuppression arm against which the other therapies is evaluated and is acknowledged to be the correct chemical immunosuppressive control for such studies i.e. CsA is considered to be the gold standard against which other immunosuppressants are tested.

RELEVANT BIBLIOGRAPHY (Reviewed key articles 1976 – 1997).

1. BROWN, J et al. Fetal pancreas as donor organ. World Journal of Surgery 1984; 8: 152–157.

2. SUTHERLAND, DER et al. Infant human pancreas. A potential source of islet tissue for transplantation. Diabetes 1976; 25: 1123.

3. MANDEL, TE et al. Organ culture of foetal mouse and foetal human pancreatic islets for allografting. Diabetes 31 (suppl. 4) 1982; 39.

4. HEGRE, OD et al. Transplantation of the fetal rat pancreas. Quantitative morphological analysis of islet tissue. Anat Record 1976; 185: 209-222.

5. Mc EVOY, RC et al. Syngeneic transplantation of the fetal rat pancreas. Diabetes 1979; 28: 141–146.

6. KORSGREW, O et al. Reinnervation of transplanted islets. Transplant Proc 1992; 24: 1025–1026.

7. MANDEL, TE et al. Organ cultured fetal pancreas. A source of islets for transplantation in diabetic mice. Transplant Proc 1981; 13: 832.

8. HOFFMAN, L et al. Insulin secretion by human foetal pancreas in organ culture. Diabetologia 1982; 23: 426.

9. MULLEN, YS et al. Complete reversal of experimental diabetes mellitus in rats by a single fetal pancreas. Science 1977; 195: 68.

10. LIKE, AA et al. Embryogenesis of the human pancreatic islets: A light and electron microscopic study. Diabetes 1972; 21(suppl.): 511–534.

11. LACEY, PE. Treating diabetes with transplanted cells. Scientific American 1995; 40–46.

12. International Islet Registry 1995; 5: 1–27.

13. LAFFERTY, KJ et al. Fetal pancreas transplantation for treatment of IDDM patients. Diabetes Care 1993; 6: 383–386.

14. LAFFERTY, KJ et al. Is there a future for fetal pancreas transplantation? Transplant Proc. 1989; 21: 2611–2613.

15. DAFOE, DC et al. An innovative site for fetal pancreas transplantation in rats – the subserosa of a U-loop of small intestine. Transplantation 1989; 48: 863–865.

16. ROBERTSON, RP et al. Effect of human fetal pancreas transplantation on secretion of C-peptide and glucose tolerance in Type –1 diabetics. Transplant Proc. 1987; 19: 2354–2356.

17. TAURA, Y et al. Fetal pancreas transplantation in miniature swine. Transplantation 1990; 50: 136–140.

18. ADAMS, GA et al. Insulin-like growth factor-1 promotes successful fetal pancreas transplantation in the intramuscular site. Surgery 1994; 116: 751–757.

19. BOULEWARE, SD et al. Diverse effects of insulin-like growth factor 1 on glucose, lipid and amino acid metabolism. American Physiological Society 1992; E130–E133.

20. UNTERMAN, TG et al. Circulating levels of insulin, insulin-like growth factor-1 (IGF-1), IGF-II, and IGF-binding proteins in the small for gestational age fetal rat. Endocrinology 1993; 132: 327–335.

21. RENNET, NJ et al. Insulin-like growth factor-1 inhibits glucose stimulated insulin secretion but does not impair glucose metabolism in normal humans. Jnl Clin Endocrinol Metab 1993; 76: 804–806.

22. THOMAS, FM et al. Insulin-like growth factor-1 and more potent variants restore growth of diabetic rats without including all characteristic insulin effects. J Biochem 1993; 291:781–786.

23. ZENOBI, PD et al. Effects of insulin-like growth factor1 on glucose tolerance, insulin levels and insulin secretion. Jnl Clin Invest 1992; 89: 1908–1913.

24. USALA, AL et al. Treatment of insulin resistant diabetic ketoacidosis with insulin-like growth factor 1 in an adolescent with insulin dependent diabetes. New England Jnl. of Med 1992; 327: 853–857.

25. ALISON, MR et al. Growth factors and growth factor receptors. Br Jnl Hosp Med 1993; 49: 774–788.

26. WESTERMARK, B. Editorial on growth factors. Cell biology International 1995; 19: 353.

27. WEBB, M et al. Inhibition of mixed lymphocyte response by monoclonal antibody specific for rat T lymphocyte subset. Nature; 1979; 282: 841.

28. MANNIE, MD et al. Differentiation of Encephalitogenic T Cells Confers Resistance to an Inhibitory Anti-CD4 Monoclonal Antibody. J Immunol; 1993; 151: 7293.

29. PELEGRÍ, C et al. Administration of a Nondepleting Anti-CD4 Monoclonal Antibody (W3/25) Prevents Adjuvant Arthritis, Even upon Rechallenge: Parallel Administration of a Depleting Anti-CD8 Monoclonal Antibody (OX8) Does Not Modify the Effect of W3/25. Cell Immunol 1995; 165: 177.

30. SIEGLING, A et al. A Nondepleting Anti-rat CD4 Monoclonal antibody that Suppresses T Helper 1-Like but not T Helper 2-Like Intra-graft Lymphokine Secretion Induces Long-term Survival of Renal Allografts. Transplantation. 1994; 57: 464.

31. ONODERA, K et al. Induction of "Infectious" Tolerance to MHC-Incompatible Cardiac Allografts in CD4 Monoclonal Antibody-Treated Sensitized Rat Recipients. J Immunol 1996; 157: 1944.

32. GUYMER RH, MANDEL TE. Urocanic Acid as an Immunosupressant in Allotransplantation in Mice. Transplantation 1993; 55: 36.

33. STUMBLES, P et al. Activation of CD4+ T Cells in the Presence of a Nondepleting Monoclonal Antibody to CD4 Induces a Th2-Type Response In Vitro. J Exp Med 1995; 182: 5.

34. PUNT, JA et al. $CD4^{+}CD8^{-}$ Thymocytes Dominate the Fetal Thymus Treated with a Combination of Anti T-Cell Receptor-ß and anti-CD4 Antibodies. J Immunol 1993; 151: 1290.

35. SAITOVITCH, D et al. Kinetics of Induction of Transplantation Tolerance with a Nondepleting Anti-CD4 Monoclonal Antibody and Donor-specific Transfusion Before Transplantation. Transplantation 1996; 61: 1642.

36. WALD, JA et al. Prednisolone pharmacodynamics: leucocyte trafficking in the rat. Life Sciences 1994; 55(19): 371-378.

37. POWRIE, F et al. $OX22^{high}$ $CD4^{+}$ T cells induce wasting disease with multiple organ pathology: prevention by the $OX\ 22^{low}$ subset. J Exp Med 1990; 172: 1701-1708.

38. MASON, D et al. Memory $CD4^{+}$ T cells in man form two distinct subpopulations, defined by their expression of isoforms of leucocyte common antigen, CD45. Immunology 1990; 70: 427-433.

39. WEIR, GC et al. Perspectives in diabetes: Scientific and political impediments to successful islet transplantation. Diabetes 1997; 46: 8, 1247–1256.

40. LA PORTE, RE et al. Preventing insulin dependent diabetes mellitus: the enviromental challenge. B Med Jnl 1987; 295: 479–481.

41. WORLD HEALTH ORGANIZATION. Diabetes Mellitus. Report of the WHO study group. Technical Report Series 727. WHO Geneva 1985; 13–45.

42. WATKINS, PJ. Diabetes and its management. Fourth Edition, Blackwell Scientific Publications 1993.

43. TARN, AC et al. Predicting insulin-dependent diabetes. Lancet 1988; I: 845–850.

APPENDIX B

Guidelines on Ethics for Medical Research

Revised edition, 1993. Based with permission extensively on Reports from the Royal College of Physicians of London

USE OF ANIMALS IN BIOMEDICAL RESEARCH

1. Introduction

The optimal care of animals used in biomedical experimentation is vital in the interests of both the animals and the research itself. There are two main reasons for this statement. Firstly, it is fitting for a civilised community to consider the humane aspects. Secondly, it must be remembered that trials involving animals are generally very expensive, and consequently the use of animals which are housed in poor facilities, which suffer from diseases or are infested with parasites, and which are badly cared for, will lead to results which are unreliable and/or unrepeatable. Good care improves the welfare of the animals and makes a large contribution to the attainment of high ethical and humane standards.

The MRC therefore wishes to:

- emphasise that the use of animals has made a large contribution to the welfare of both humans and animals in the past, and that future use of animals is necessary for further progress;
- acknowledge that humans have an ethical duty to treat all animals with great care and to be aware of their susceptibility to pain and suffering; emphasise that experimentation with a wide variety of animal species is necessary for the advancement of knowledge in biology, as well as the development of methods for prevention, diagnosis and treatment

of diseases of humans and animals and the advancement of their welfare and productivity; support the responsible use of animals for experimentation and other scientific purposes, but encourage the use of valid alternative methods, where possible;

- insist that complete care be taken to protect animals from pain, suffering, discomfort and permanent injury, and will ensure that in cases where it is unavoidable, it will be kept to a minimum;
- ensure compliance with all relevant South African legislation, the National Code for the use of animals in research and international regulations. These include the following:
- the Animal Protection Act (Act No. 71 of 1962);
- the Animal Diseases Act (Act No. 35 of 1984);
- the National Parks Act (Act No. 57 of 1976);
- the Nature Conservation Ordinances of the four provinces (Cape Province - Ordinance No. 19 of 1974; Orange Free State - Ordinance No. 8 of 1969; Natal - Ordinance No. 15 of 1974; Transvaal - Ordinance No. 12 of 1983); the National Code for the Handling and Use of Animals in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa; and Convention on International Trade in Endangered Species (CITES).

2. Definitions

- `Experimental animal' means any living non-human vertebrate, non-human vertebrate fetus, or any other animal species which in the opinion of the Ethics Committee for Research on Animals (ECRA), has a nervous system which is so sophisticated that it might be capable of experiencing pain in much the same way that any vertebrate might experience it.
- An `animal experiment' is any procedure involving the use of live animals in which the aim is to test a hypothesis, collect information and advance, impart or demonstrate knowledge, test or collect a product, or register the effect of a certain procedure on an animal.

Prerequisites for the use of animals in research

- The main purpose of any animal experimentation must be to gain usable results and scientific information of high quality to the benefit of humans and animals. The experimentation must therefore not be purposeless or unnecessary.
- The experiment must be carefully and scientifically planned, based on the available knowledge of the disease or problem being studied and designed so that the expected results, wherever possible, will justify the experiment. A written protocol should therefore be prepared before each experiment which clearly indicates the purpose of the experiment as well as all procedures to be carried out. No experiments on animals must be allowed to start before the research protocol has been passed by the Research Committee of an institution and the ECRA has separately evaluated and passed the experiments.
- All attempts should be made to keep the use of animals to a minimum. Care should be taken to ensure that the correct species is chosen and that animals with all the necessary genetic attributes and microbiological qualities are chosen to ensure reliable results.
- The application of in vitro biological systems, statistical analyses and mathematical models should be considered as alternatives to supplement or replace animal experimentation. These alternatives must not only be considered on the grounds of humane principles but also because they generally demand less time, space, equipment and funding. Where the experiment inflicts inescapable pain or more pain than the use of anaesthetics would cause, the proper use of analgesics or the administration of anaesthesia according to recognised veterinary practice is obligatory until the procedure is completed. The only exception to this principle is in cases where the administration of anaesthesia would nullify the aims of the experiment and the results could not be obtained by any other more humane method. If approved, the Committee must then appoint a veterinarian to take responsibility for the welfare of the animals subjected to those procedures. As a general guide, researchers should accept that any procedure which will cause pain to humans will also cause pain to other vertebrates.
- The scientist in charge of any animal experiment must be prepared to terminate it if it becomes clear that the continuation thereof will cause unwarranted pain and suffering. If the procedure causes serious injury, the animal should be killed before recovery from

anaesthesia. If it becomes clear that an animal will suffer unwarranted pain or discomfort after an experimental procedure, it should be killed in a humane manner. The veterinarian must have an overriding discretion as to when animals shall be killed or withdrawn from an experiment for humane reasons.

- The clinical care of experimental animals before, after and during the application of the experimental procedure must be of high standard and according to accepted veterinary practice so that pain, discomfort and any detrimental effects, caused by the procedure, can be eliminated. If it becomes necessary to kill an experimental animal it must be done in a humane manner and according to accepted principles which ensure immediate death. It is preferable that only personnel with experience in the application of euthanasia are responsible for this task.
- No animal may be disposed of before there is absolute certainty that it is dead.
- Animals already used in an experimental procedure should not be subjected to it for a second time, unless the first procedure was harmless or non-invasive and left the animal in good health.
- No animal should be subjected to more than one procedure that causes significant pain.

Care

- The care of experimental animals should be under the direct control and supervision of a veterinarian, preferably one with experience in animal experimentation, and a qualified laboratory animal technologist. The supervisory personnel should also include the services of technical staff and workers with experience and/or training in laboratory animal science.
- High standards should be maintained in the daily care of experimental animals. Special consideration should be given to regular feeding, adequate and clean water, hygienic surroundings, adequate ventilation and the elimination of excessive heat, cold or noise in the animals' environment.
- Care should be taken to eliminate disease, injury, overpopulation and stress factors and to safeguard the animals from endo- and ectoparasites. Careful and thorough supervision of the welfare of all animals is absolutely essential.

- Experimental animals should be kept under optimal conditions at all times. This includes good housing, correct environmental conditions with acceptable space for movement and opportunity for social interaction except where non-compatibility or the requirements of the experiment prevent it. The animal holding cages as well as the premises in which the cages are housed should therefore meet with accepted minimum standards.
- Wild animals which are captured in their natural habitat, for research purposes, should be trapped in cages that meet with the standards of the nature conservation authorities. The traps should be visited regularly, preferably daily, to prevent animals being left without food and water for long periods.
- It is the duty of the head of the research institute to ensure that all researchers who use experimental animals have the necessary training and experience to do so correctly. In-service training programmes should be established at research institutes to ensure that this requirement can be met.

Transport

- Experimental animals must be transported according to the recognised minimum standards and regulations for the transportation of animals.
- During transportation special care should be taken for the provision of good ventilation, the elimination of discomfort, excessive cold or heat and the spread of diseases.
- Provision must be made during long journeys for regular provision of food and water.
- Animals must be unloaded as quickly as possible on arrival at an airport, harbour or railway station. At their ultimate destination they must be removed from their transport cages immediately and placed in suitable permanent accommodation.
- Appropriate veterinary care must be given to animals found to be diseased, injured or in a poor state during travel or on arrival.

Ethics Committee for Research on Animals (ECRA)

- All institutions using animals in experimentation must establish an ECRA. No animal experiment may be permitted to start before the research protocol has been passed by both the ECRA and the Research Committee of the institution.

- The objective of an ECRA is to control the use and care of experimental animals. The ECRA must be properly constituted, ensure that these guidelines are followed and applied, keep records of the type and number of animals used in each experiment and ensure that quantifiable norms and standards are adhered to.
- An ECRA must be constituted by and shall report to the management of the research institution.
- The ECRA shall preferably include persons from each of the following categories as members:
- Established researchers who have experience of the use of animals in experiments.
- A registered veterinarian.
- Persons not engaged in research representing recognised animal welfare organisations and appointed by mutual agreement between the institution and the organisation.
- Persons not engaged in research and not associated with the institution or a recognised animal welfare organisation.

The responsibilities of an ECRA are:

- to establish an institutional code of practice for the use and care of experimental animals and to ensure that all proposed procedures comply with this code and meet accepted ethical, legal and scientific requirements;
- to maintain a register with particulars of the species, number and origin of the animals used as well as the type of experiments done;
- to inspect the animal facilities regularly and without warning;
- to only approve an animal experiment if convinced that it is justified and feasible, if not, the ECRA must refer the matter back to the Research Committee or to subject-matter experts who are not members of the ECRA;
- to preview and review all animal experiments in the institution;

- to ensure that the species chosen is the best model and that no valid alternative method can be applied;
- to ensure that pain, discomfort, stress or distress is minimised or eliminated;
- to ensure that experiments do not exceed preset time limits, and that avoidable delays do not occur;
- to ensure that all experimental procedures and related activities done on animals are performed by persons with the necessary experience and skills; and
- to ensure that its decisions are carried out, because the final responsibility for the welfare of experimental animals lies with the Committee.

The duties of the ECRA shall be to create mechanisms that provide for:

- regular meetings;
- the stringent review of experiments;
- acceptable standards of clinical care of animals;
- acceptable standards of husbandry care of animals;
- the immediate termination of experiments in which the animal is suffering unacceptable or uncontrollable pain, in the view of the veterinarian; and
- to report regularly to the management of the institution.

Special provisions in respect of standards and norms

Organisations using experimental animals should accept and apply quantifiable norms and standards. For this purpose, the proposals of recognised national and international authorities, advisory bodies and other authorised organisations should be accepted, in order to make provision for:

- the appointment of ECRAs;
- the procurement of experimental animals;
- correct transportation of animals;

- adequate accommodation and animal care;
- optimal environmental conditions;
- correct feeding;
- thorough veterinary care and treatment;
- the keeping of thorough records; and
- euthanasia.